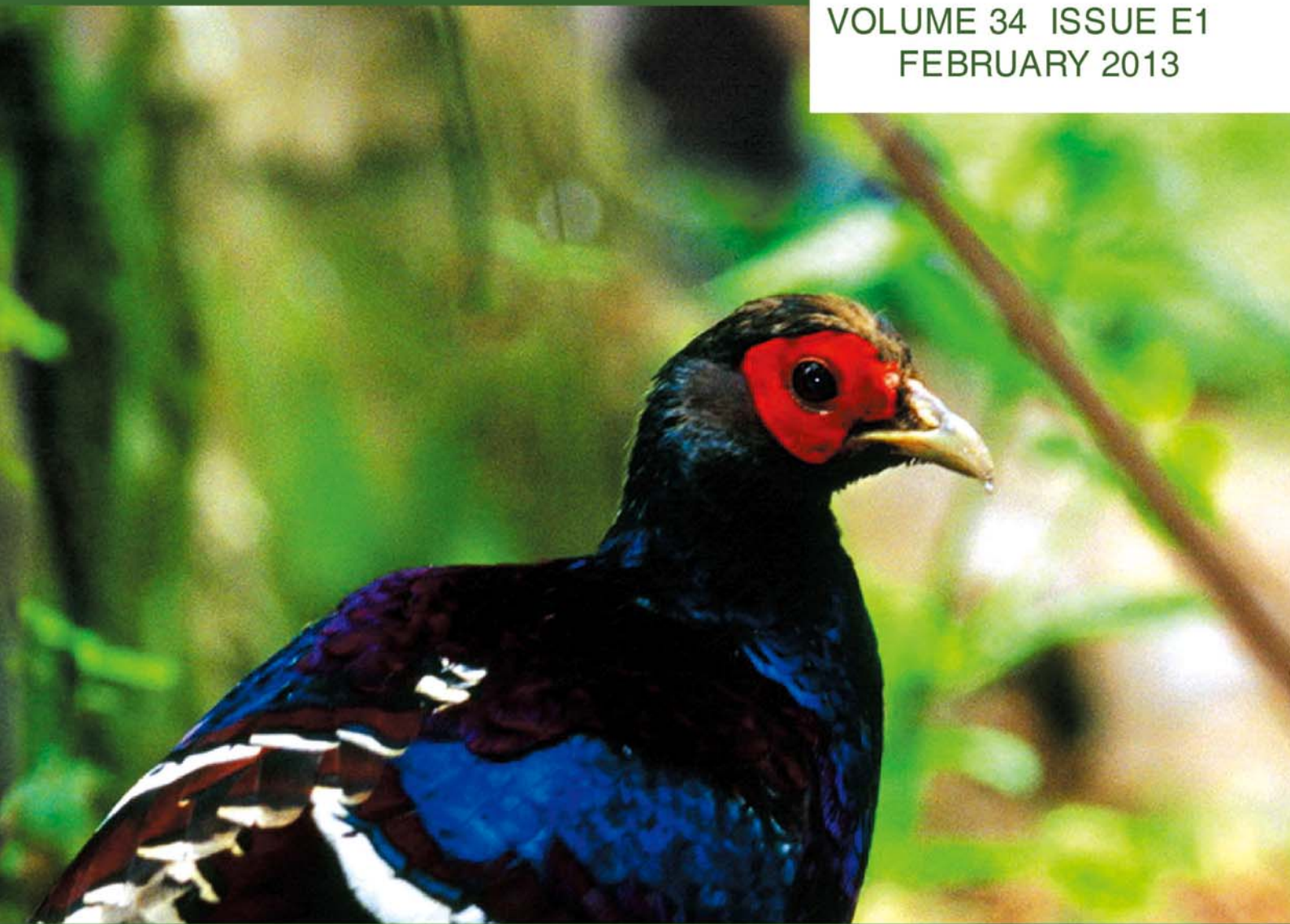


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Proximity association in polygynous western black crested gibbons (*Nomascus concolor jingdongensis*): network structure and seasonality

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Abstract: We investigated the structure and seasonality of the proximity network in a group of polygynous western black crested gibbons (*Nomascus concolor*) using social network analysis. The spatial proximity changed seasonally and was affected by temperature and rainfall. Preferred proximity association was not distributed randomly among individuals. Kinship was one explanation for the social structure, as offspring preferred to maintain close proximity with their mothers. The proximity of infants to mothers decreased with age, and independent offspring had lower proximity to mothers than dependent ones. We found that the adult male had different proximity relationships with two different adult females. The frequency of proximity between the male and the infant-carrying female was significantly higher than that between the male and the female who had immigrated carrying one offspring of uncertain paternity into the group. Infanticide avoidance and/or predation protection for dependent infants might explain the proximity relationship differences. Temperature influenced group proximity association, with individual proximity increasing in the cold months and decreasing in the hot months. Group proximity decreased in months with higher anthropogenic disturbance.

Keywords: Proximity; *Nomascus concolor*; Polygynous; Social network

Spatial distance between individuals exists in all group living animals; however, variances occur among different species and among individuals within the same group. It is reported that spatial distance among individuals is correlated with social relationships (Sade, 1965, 1972) and social organization (Kummer, 1968); therefore spatial proximity between group members is important for studying social structure. Early research reported on a correlation between inter-individual distance and social relationship in *Macaca mulatta* (Sade, 1965), with further studies using spatial data to represent social structure in different primates (e.g. *Symphalangus syndactylus*: Chivers, 1971; *Papio cynocephalus* and *Saimiri sciureus*: Fairbanks, 1976). In recent years, spatial distance has also been used in species with different social structures, such as multi-male-multi-female groups (e.g. *Macaca mulatta*: Sherman, 1980; *Macaca nemestrina* and *Macaca arctoides*: Weigel, 1980; *Macaca fuscata fuscata*: Corradino, 1990) and multilevel society groups (e.g. *Theropithecus gelada*: Snyder-Mackler et al, 2012; *Papio hamadryas*: Schreier &

Swedell, 2012; Swedell & Plummer, 2012; *Rhinopithecus roxellana*: Zhang et al, 2012).

Social structure in animal groups can be affected by many factors, such as kinship (Furuichi, 1984; Matsumura & Okamoto, 1997; Silk, 2002), age classes (de Waal & Luttrell, 1986), resource holding potential (Barrett & Henzi, 2006), and resource availability (Henzi et al, 2009). A number of studies have also investigated the relationship between ecological factors and social behavior (Emlen & Oring, 1977; Terborgh, 1986; Sterck et al, 1997; Kappeler & Van Schaik, 2002) and shown that social relationships may change in different seasons (Shimooka, 2003) and habitats (Pruetz & Isbell, 2000). However, compared to numerous studies on the effects of seasonal ecological factors on animal behavior (Dunbar,

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2002; Dunbar *et al.*, 2009), such as activity budgets (e.g. *Theropithecus gelada*: Dunbar, 1992; *Ateles* spp.: Korstjens *et al.*, 2006; *Nomascus concolor*: Fan *et al.*, 2008; *Eulemur fulvus fulvus*: Sato, 2012; *Nomascus nasutus*: Fan *et al.*, 2012) and range behavior (e.g. *Theropithecus gelada*: Dunbar, 1998; *Symphalangus syndactylus*: Kim *et al.*, 2011), how social relationships are affected by ecological factors is not well understood, with very few studies conducted on small apes.

Social relations are not easily investigated (Whitehead, 1997), and the development of appropriate measures for spatial proximity can express qualitative variation and assess the social structures (Cairns & Schwager, 1987; Whitehead, 1995; Ramos-Fernández *et al.*, 2009). Early research on spatial distance in siamangs (*S. syndactylus*: Chivers, 1971) considered spatial relationship as an important measure for group cohesion. However, few studies have addressed social relationships or spatial proximity in gibbons. While most gibbon species live in small monogamous groups (Leighton, 1987), recently several gibbon species have been found to live with flexible group composition (Brockelman, 2009). Lappan (2007) used spatial distance to study social relationship among adults in multi-male siamang groups (*S. syndactylus*). Stable multi-female groups only exist in the black crested gibbon (*Nomascus concolor*: Jiang *et al.*, 1999; Fan *et al.*, 2006), Hainan gibbon (*N. hainanus*: Zhou *et al.*, 2005) and Cao Vit gibbon (*N. nasutus*: Fan *et al.*, 2011). Polygynous groups generally have more individuals (Jiang *et al.*, 1994b; Fan *et al.*, 2006; Zhou *et al.*, 2008), and studying social relationships in such groups (e.g. *N. concolor*: Fan & Jiang, 2009) can aid understanding of the social system evolution mechanism in gibbons. However, no research on the influence of ecological factors to spatial proximity in gibbons has been conducted to date, though Fan *et al.* (2008, 2012) found black crested gibbons and Cao Vit gibbons were more likely to stay together in cold seasons during the night.

Our aim was to study proximity associations in a polygynous group of western black crested gibbon in Mt. Wuliang, central Yunnan, and assess the impacts of ambient temperature and rainfall on seasonal variance. We also considered the influence of anthropogenic disturbance on proximity among group members.

METHODS

Study site and subjects

We carried out the study at Dazhaizi (N24°21', E100°42') on the western slopes of Mt. Wuliang, in Jingdong County, central Yunnan, China. We used a HOBO pro RH/Temperature Data Logger (RG3-M) to record the temperature every two minutes and rainfall continuously in the study area from January 2011 to

December 2011. Our study group was habituated in December 2010 after thirty months of previous following. The group was composed of seven individuals: one adult male, two adult females, one sub-adult male, two juveniles, and one infant (Table 1). All individuals were recognized by the observers according to their facial features and body size. We named the individuals of the group following Guan *et al.* (submitted) (Table 1). InR was born in November 2009 and was still dependent on its mother. JuI was born in 2008 and JuR was born in 2006, both were fully independent.

Table 1 Composition of the study group

Adult male	Sub-adult male	Adult female	Juveniles	Infant
AM2	SAM2	AFR	JuR (offspring of AFR)	InR (offspring of AFR)
		AFI	JuI (offspring of AFI)	

Data collection

We spent approximately 20 days per month in the field. Every morning the group was located by listening for their morning duets, going directly to their sleeping sites, or by visiting their food patches. Once the group was located, we followed it as long as possible and used scan sampling at 5-min intervals to collect data on the spatial distance among all independent individuals, with spatial proximity defined as individuals within 1 m of each other at the time of scan sampling. For collection of distance data, we estimated distance between any two trees by eye and confirmed it using a direct-reading optical range finder (OLC 600XV) in 2010. We achieved a distance estimation of <10 m with error <1 m and 10-20 m estimation with a ca. 2 m error. We then collected systematic data between January 2011 and December 2011.

We considered one observing day as effective only if the group was followed for more than 5 h in that single day, and only data collected on effective observing days were included in the following analysis.

Data analysis

Simple ratio association index (hereafter SRI) was used to study pairwise associations. The SRI was calculated using the number of scans in which two individuals were seen in proximity divided by the total number of scans during which the dyad was in proximity and was separated (Cairns & Schwager, 1987):

$$SRI_{AB} = X_{AB} / (X_{AB} + Y_{AB}), \text{ and } Y_{AB} = y_A + y_B + y_{AB}$$

In the formula, X_{AB} stands for the number of scans in which A and B were seen in proximity while Y_{AB} means they were not; y_A and y_B mean the number of samplings when only A was located or only B was located separately; and y_{AB} represents the number of scans in which both A and B were located but were not in proximity. For each pair, total observation time varied among months. To avoid the influence of different

sampling size, we first calculated monthly SRI and then averaged them to obtain annual mean SRI for every dyad.

Social network analysis (hereafter SNA) is a useful tool for studying social structure in different primates (Kasper & Voelkl, 2009; Whitehead, 2009). In this paper, SNA was used for studying the proximity network, which was built in Ucinet 6.0 (Borgatti et al, 2002). We first constructed a *weighted* proximity network using annual SRI of each dyad, which represented the presence or absence as well as the frequency of proximity distance. We used *group density* as a measure of group cohesion, which was defined as the number of observed dyads with proximity divided by the number of possible dyads according to the number of individuals in the network (Hanneman & Riddle, 2005). To highlight the more important dyads in the network, we then defined preferred proximity dyads as those with SRI higher than or equal to the mean SRI of all possible dyads (Kanngiesser et al, 2011). We built an *unweighted* network by the transformation of SRI into binary values with 1 standing for the preferred proximity dyads and 0 standing for the non-preferred ones, i.e., only preferred proximity dyads were retained in this network. This approach has not been well validated in former studies, so we also used quadratic assignment procedure (QAP) to test whether the *unweighted* network adequately reflected the structure of the *weighted* network. Bootstrap replicates were used to compare the density of preferred proximity networks to a theoretical fully connected network (density=1) to investigate whether preferred proximity was distributed randomly.

We conducted hierarchical cluster analysis in Socprog 2.4 (Whitehead, 2009) to assess the associations among hierarchically formed clusters. Cophenetic correlation coefficient indicated the fitness of the dendrogram as representative of the observed data. Cophenetic correlation coefficients > 0.8 (Whitehead, 2009) indicate a good match. We used a paired sample test to investigate whether the adult male had different proximity association with the two females and their offspring by comparing their monthly SRI. Kolmogorov-Smirnov test was used for normality before we made comparisons of different dyads. Paired sample test was also used to compare the proximity association of one female and her two offspring of different ages. To access the trend of proximity association change of mother-offspring dyads, we used multiple linear stepwise regression to test the impact of month (i.e. when the juveniles or infant matured) and environment factors (temperature and rainfall). To describe the variance of seasonal social proximity, we calculated the mean SRI of the group in twelve months, and used linear stepwise regression to test the impact of temperature and rainfall. Because the group was newly habituated in January 2011 and was not as well observed as in the other eleven

months, we removed the January data and estimated the influence of weather again. All statistical analyses were conducted via SPSS 16.

RESULTS

The mean temperature during the study period was 15.7 °C, with the lowest mean in January (10.1 °C) and the highest in June (19.2 °C). Precipitation was 1793 mm from January 2011 to December 2011, with an obvious wet season from May to October during which 84% of the rainfall occurred (Table 2).

Table 2 Seasonal variation of monthly mean SRI and group density from January to December in 2011

Month	Mean SRI	Group density	Mean temp. (°C)	Rainfall (mm)
Jan	0.1160	0.4762	10.1	35
Feb	0.2008	0.8095	13.5	0.2
Mar	0.1512	1.0000	14.4	65.8
Apr	0.1177	0.8095	16.9	89.4
May	0.0537	0.7619	18.2	115.8
Jun	0.0616	0.7619	19.2	314
Jul	0.0608	0.8095	18.9	382.6
Aug	0.0520	0.7143	19.0	258.8
Sep	0.1139	0.7143	19.1	318.2
Oct	0.1276	0.9048	15.6	114
Nov	0.1662	0.8571	12.1	69.4
Dec	0.1957	0.9524	11.1	29.8

During our study period, 5229 scans were made and 31013 distance data were collected. Group density varied monthly. The group was cohesive (group density > 0.7) in all months except January (lowest density value of 0.48, Table 2). However, annual group density was 1 (fully connected) for the *weighted* network as all possible dyads were observed in proximity ($n=21$). The preferred proximity (*unweighted*) network well reflected the structure of the weighted network (QAP, $R=0.609$, $P=0.001$). A preferred proximity relationship only existed in about half of all possible dyads (density was 0.4762 for this network, i.e. 10 preferred proximity dyads in 21 possible pairs) and preferred proximity was not distributed evenly (Bootstrap test: $z = -3.0755$, $P=0.0002$). The individual SAM2 exhibited no preferred proximity with other members, which confirmed his peripheral position (Figure 1).

Using hierarchical cluster analysis, we identified four subgroups with a cophenetic correlation coefficient of 0.98: the two females with their own offspring, the adult male (AM2), and the sub-adult male (SAM2). Results showed that AM2 preferred proximity with AFR and her offspring more often than with AFI and her

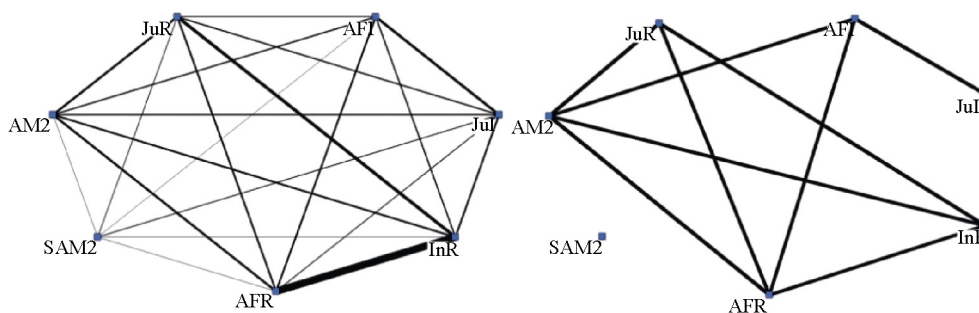


Figure 1 Weighted (left) and unweighted (right) social network of proximity (using Ucinet 6.0)

Nodes represent individuals and lines represent the presence of association between individuals. In the weighted network, the thickness of the line represents the frequency of proximity position.

offspring (Paired sample test: AM2-AFR vs AM2-AFI, $df=11$, $t=3.048$, $P=0.011$; AM2-InR vs AM2-Jul: $df=11$, $t=3.230$, $P=0.008$; AM2-JuR vs AM2-Jul, $df=11$, $t=2.274$, $P=0.044$) (Figure 2).

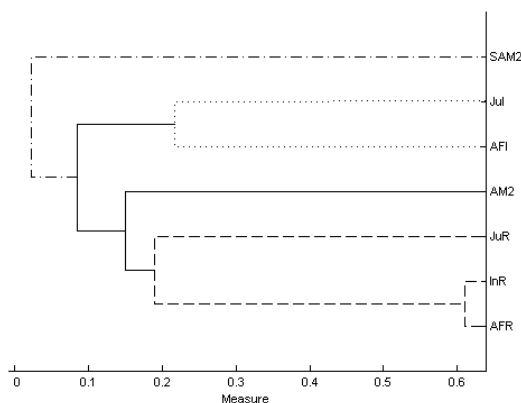


Figure 2 Dendrogram of proximity associations between individuals created in SCOPROG 2.4 (Whitehead, 2009)

Labels on the right axis represent the name of individuals. Four subgroups are identified by different line types.

Three mother-offspring pairs were compared, and all SRI data were normalized distributed (Kolmogorov-Smirnov test: AFR-InR, $n=12$, $P=0.657$; AFR-JuR, $n=12$, $P=0.352$; AFI-Jul, $n=12$, $P=0.943$). The SRI between mother and dependent offspring (AFR-InR) was significantly higher than between mother and independent offspring (AFR-JuR) (Paired samples test: $df=11$, $t=7.161$, $P=0.000$), but no significant variance was observed between two independent offspring (Paired samples test: AFR-JuR vs AFI-Jul, $df=11$, $t=-2.048$, $P=0.065$). Stepwise regression showed that the proximity of the dependent infant with their mother was only affected by age, that is, proximity decreased with age (InR-AFR, $F_{1,10}=36.992$, $r^2=0.787$, $P=0.000$, Figure 3). However, the SRI of fully independent offspring and their mother was significantly affected by mean

temperature (Jul-AFI, $F_{1,10}=6.205$, $r^2=0.383$, $P=0.032$) and rainfall (JuR-AFR, $F_{1,10}=5.014$, $r^2=0.334$, $P=0.049$). The group's mean SRI changed monthly (Table 2) and ambient temperature significantly influenced proximity association ($F_{1,10}=14.404$, $r^2=0.591$, $P=0.004$). After removing January data, the impact of temperature increased ($F_{1,9}=42.315$, $r^2=0.825$, $P=0.000$). The SRI increased in the cold months, with the highest in February and December (0.20). The group maintained low proximity association while the mean temperature was high from May to August, with the lowest SRI (0.05) appearing in May and August (Figure 4).

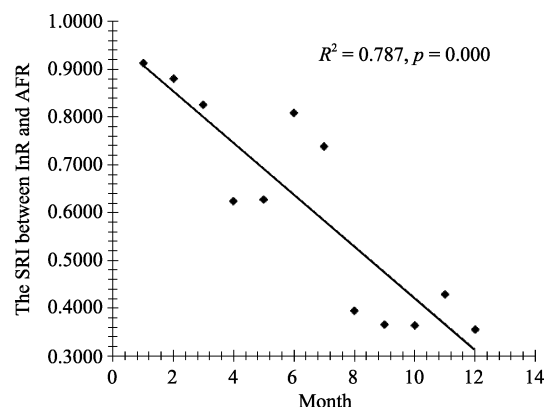


Figure 3 SRI between mother and dependent offspring (AFR and InR)

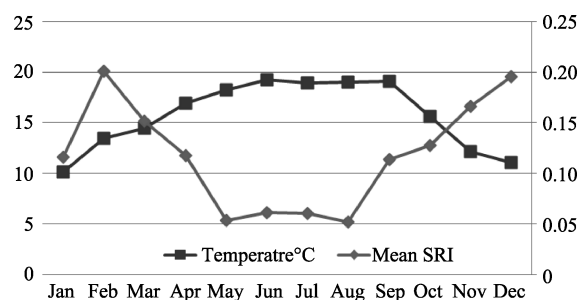


Figure 4 Correlation between mean temperature and group mean SRI

DISCUSSION

Social structure of a polygynous group of gibbons

Kasper & Voelkl (2009) investigated social cohesion in 70 primate species, and found a mean density of 0.75 ($n=70$, range 0.49–0.93). Gibbons live in small groups. Although the claim of nuclear family groups may not be correct in many gibbon species, and adult replacement as well as extra-group copulation have been observed in our study group (Fan et al, 2010; Huang et al, accepted), this group still showed a fully connected network (density=1), which meant all individuals directly connected with each other and represented very high social cohesion. This may be how gibbons maintain group stability, especially when paternity is uncertain. However, the proximity association between different individuals was uneven. Only ten dyads exhibited preferred proximity, and the sub-adult (SAM2) obviously exhibited peripheral clustering in the study group, as has been shown in previous research (Fan & Jiang, 2009). Four clustering units were identified and kinship was a good explanation for the observed pattern (Furuichi, 1984; Matsumura & Okamoto, 1997), as females preferred to keep proximity with offspring. Even among young gibbons, individuals also maintained proximity with individuals of the same matrilineal line. When the dependent offspring grew older, the proximity between mother and offspring declined, as observed in saki monkeys (*Pithecia pithecia*: Thompson & Norconk, 2011).

In our study group, the most notable characteristic was that the adult male had different proximity association with the two adult females. This differs from previous research, which reported that two females maintained similar spatial distance from the adult male in the neighboring group (Fan & Jiang, 2009). This variance may be due to different analysis methods as we defined 1 m as a proximity distance while Fan & Jiang (2009) used mean spatial distance for measuring the relationship. Considering the group history and different reproductive status of the two females (infant-carrying or not), we can give two possible explanations for our results.

Previous research has shown infanticide affects social structure (Sterck et al, 1997). Reichard (2003) argued that infanticide is a potential threat for newborns and also concluded that infanticide avoidance could drive the evolution of monogamy in gibbons, suggesting females also face infanticide stress. In 2008, AFI and her newborn infant (JuI) immigrated into the study group (Huang et al, accepted; Guan et al, submitted). This female was never seen previously in the study area, and the paternity of the infant was unknown. Such circumstances increase the risk of infanticide. Although infanticide has never been reported in gibbons, with the possible exception of one case in Khao Yai (Reichard,

2003), we considered that infanticide avoidance may be one reason for AFI to limit proximity with AM2. The adult male AM2 grew up in the neighboring group and immigrated into our study group in 2007, replacing the original adult male (Fan et al, 2010). Thus we cannot be sure whether AM2 is or is not the biological father of any young within the group (InR and JuI). Genetic data would help solve this uncertainty.

Alternatively, infants may affect the relationship between males and females because male proximity is important to protect a dependent infant from predation (Dunbar & Dunbar, 1980). This has been reported in small primates such as titis monkeys (*Callicebus discolor*: de Luna et al, 2010) and saki monkeys (Thompson & Norconk, 2011), as well as in white-handed gibbons (*Hylobates lar*: Reichard, 2003) and siamangs (*Symphalangus syndactylus*: Lappan, 2008). In our study area, gibbon infants face predation from eagles (*Spilornis cheela*) (personal observation), and obviously dependent offspring (InR) would most likely require protection. Although prevention from predation is considered a more efficient strategy in monogamous than in polygynous groups (Sommer & Reichard, 2000), we found that the adult male kept proximity with the female carrying dependent offspring (AFR) in the polygynous group.

Long term observation is necessary to determine which explanation is more reasonable. If the male maintains greater proximity with the infant-carrying female, the latter protection hypothesis can be proved; if the proximity of AFI and AM2 increases as JuI matures, the infanticide-avoidance hypothesis would be preferred; if the male maintains proximity to AFR, the impact of individual preference or/and sibship among individuals may be considered; or, it is also possible that the male maintains proximity to one female over another randomly among years.

Seasonal variance of proximity

Temperature influences on group proximity

Many studies have reported on the influences of ambient temperature on animal behavior (e.g. *Theropithecus gelada*: Dunbar, 1998; *Symphalangus syndactylus*: Kim et al, 2011; *Eulemur fulvus fulvus*: Sato, 2012; *Nomascus concolor jingdongensis*: Fan et al, 2008). In our study, we found that temperature significantly influenced group proximity, with individuals spending more time in spatial proximity when the temperature was low. This phenomenon was likely a response to thermoregulatory needs, which is an important ecological force (Hill et al, 2004). Gibbons in Mt. Wuliang inhabit the northern extreme of Hylobatidae habitat and are at high altitude with extreme seasonality of temperature and rainfall, resulting in great challenges associated with thermoregulatory costs. Previous studies have shown that both black crested gibbons in Mt.

Wuliang (Fan & Jiang, 2008) and Cao Vit gibbons in Guangxi huddled together in their sleeping places in cold months while they slept separately or apart in the warmer months (Fan *et al.*, 2012). Our study showed that gibbons also increased time in proximity through the day time in cold months.

According to our results, group proximity decreased regularly from the dry season to the rainy season and reached the lowest point from May to August. This may relate to the seasonal time budget in the study subjects. Food availability increased in the rainy season and the gibbon group increased feeding time and decreased resting time (Huang, 2011). The gibbons seldom stayed at proximity distance when feeding, singing, and moving, with most proximity/touching occurring during resting time. Therefore, when the group decreased resting time, they also decreased the time spent in proximity distance.

Human activity influences on group proximity

Anthropogenic disturbance might also influence group proximity. In social animals, an individual's fitness depends partly on the quality of relationships with others (Schülke *et al.*, 2010; Silk *et al.*, 2010; Seyfarth & Cheney, 2012). Good social relationships provide partners with direct benefits such as grooming and support in aggressive conflicts, or increase tolerance near resources and, thus, decrease competition (Silk, 2007; Massen *et al.*,

2010; Seyfarth & Cheney, 2012). However, these benefits might disappear when the group meets severe disturbance.

In a previous study, Fan & Jiang (2010) found gibbon behavior changed in high human disturbed areas. Most human activity (livestock grazing) in the study area was found at low altitude (Fan & Jiang, 2010). The low proximity from May to August may be due to high frequency of human disturbance. In those months, more food resources were available at low altitude and mushroom collecting occurred more frequently, thus gibbons had to increase moving time and keep alert. Gibbons may increase distance between individuals, or even move in different directions when avoiding human disturbance (Jiang *et al.*, 1994a). Although gibbons spent more time in the low area from November to March, human disturbance was lower in those months. After August, gibbons spent most of the time in the high altitude area (over 2 300 m), where human activity was rarely found.

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Attacks on adult females with infants by non-resident males in the François langur

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Abstract: Infanticide by males is a common phenomenon in mammals, especially primates, as lactation lasts much longer than gestation in many species. Usually, infanticidal episodes occur soon after group takeovers, and are traditionally considered a male reproductive strategy (i.e., support the sexual selection hypothesis, Hrdy, 1974). To verify the validity of this conception, we observed one group of François' langurs in the Nonggang Nature Reserve, China, between August 2003 and July 2004. During the study period, a François' langur female with the youngest infant in the group was attacked three times by immigrating males, and later disappeared by the third day after the final attack. We suggest that these attacks on the female-infant dyad represent infanticide attempts by males, and may be the cause of the adult female and her infant's disappearance. Presumably, that female dispersed with her infant to avoid infanticide and was not killed. Though these observations do not completely verify the sexual selection hypothesis, they are not inconsistent with it.

Keywords: Infanticide; Reproductive tactics; François' langur (*Trachypithecus francoisi*)

Infanticide by males is a common phenomenon among some mammal taxa. In primates, this occurs because some species show a period of lactation longer than gestation (van Schaik & Janson, 2000). Researchers have proposed numerous hypotheses to explain the significance of infanticide, such as the sexual selection (Hrdy, 1979; van Schaik & Janson, 2000), resource competition (Agoramoorthy & Rudran, 1995), and social pathology hypotheses (Bogges, 1979). Among male infanticide in primates, sex selection is usually supported by observational data (Hrdy, 1979; van Schaik & Janson, 2000), even though this hypothesis is still occasionally debated in anthropological circles (Sussman et al, 1995; Sommer, 2000). Based on the sexual selection hypothesis, infanticide is thought to be male reproductive strategy whereby males prevent females from investing in offspring sired by other males, thus increasing the chance of siring offspring of their own (Hrdy, 1979, van Schaik & Janson, 2000).

Because detecting infanticide is difficult, many reports of infanticide in primates have been based on indirect observations (Newton & Dunbar, 1994; Steenbeek, 2000). To more directly examine if the sexual selection hypothesis bears out in certain primates, we

began observing a group of François' langurs. The François' langur (*Trachypithecus francoisi*) is an endangered colobine species, ranging from the Red River in northern Vietnam to Guangxi, Guizhou, and Chongqing in southern China (Groves, 2001; Wang et al, 1999). The langurs live in habitats characterized by Karst topography. Groups are predominantly polygynous, and range from 5–13 individuals. Their society is typically characterized by female philoparty and male dispersal (Hu, 2007; Wu, 1983).

Previously, attacks on infants after male takeovers have been seen in the white-headed langur (*T. leucocephalus*), a close phylogenetic relative living in similar habitat (Li, 2000; Zhao et al, 2011), but not in the François' langur. This behavior was considered to be infanticide, and a male reproductive strategy (Li, 2000; Zhao et al, 2011). Here, we provide data from several months of observation, documenting three cases of

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attacks on adult females with infants by immigrant adult males in a free-ranging group of François' langurs. We suggest that these attacks were attempts at infanticide and thus targeted at the infant. Our main aim here is to explore possible causes of male attacks, and evaluate the validity of the sexual selection hypothesis to explain these data.

STUDY SITES AND METHODS

Nonggang Nature Reserve is located in the south-west of Guangxi province, China (E106°42'–107°4', N22°13'–22°33'), and comprises three areas: Nonggang (5426 ha), Longhu (1034 ha), and Longshan (3949 ha), which are separated by farmlands and villages. This reserve consists of limestone hills with elevations ranging from 300 m to 700 m above the sea level (Guangxi Forest Bureau, 1993) and vegetation is characterized as seasonal rainforest (Shu et al, 1988). Our field research was carried out in the northwestern portion of Nonggang, in the main study area of about 200 ha. The study group ranged near to our camp and had been semi-habituated to observers before data collection began. The size of the group varied from 12 individuals (4 adult males, 5 adult females, and 3 immatures) at the start of the study to 10 individuals by the end, owing to the disappearance of an adult female and her infant.

Between August 2003 and July 2004, we spent a mean of 10 d each month following the focal group. On each day, we observed the langurs via binoculars (10×60) at a distance of 10–200 m. During full-day follows, we began data collection at 06:00 and ended it when the subjects entered the sleeping site. We also collected

behavior data during partial-day follows, which began when we first encountered the langurs. We collected no data in July 2004, due to widespread flooding, making follows almost impossible. We used instantaneous scan sampling in behavioral data collection, with 15 min intervals. A total of 739 h of behavioral data were collected.

Attacks were defined as the abrupt onset of an episode in an aggressive way, with or without body contact. Whenever an attack occurred, information on the attack, including time, identity of attacker and victim, the sequence, and the behavioral context, were recorded *ad libitum* (Altmann, 1974). Because observation was conducted from a considerable distance, or the event occurred at dusk, we could not identify all individuals, and accordingly only identified the age-sex classes involved. We estimated the age of infants based on the amount their pelage had changed from their natal coat color and body size.

RESULTS

Dynamics of the study group

The study group included 4 adult males at the start of this study, and group composition has changed during the study period (Table 1). Though François' langur society is typically characterized by female philoparty and male dispersal (Hu, 2007), an atypical instance of female dispersal may have occurred during our observation. An adult female with the youngest infant disappeared from the study group on 24 December, 2003 after attacks were directed towards her and her offspring (see below).

Table 1 Changes in group size and composition during study period

Time	Group size	Group composition	Note*
2003.8.13–2003.10.2	12	4 adult males, 5 adult females, 3 infants	
2003.10.4–2003.10.9	8	5 adult females, 3 infants	4 adult males left group
2003.10.10–2003.11.11	12	4 adult males, 5 adult females, 3 infants	4 adult males joined group
2003.11.25–2003.12.13	8	5 adult females, 3 infants	4 adult males left group
2003.12.14–2003.12.23	12	4 adult males, 5 adult females, 3 infants	4 adult males joined group
2003.12.24–2004.7.25	10	4 adult males, 4 adult females, 2 infants	an adult female with infant disappeared

*During the study period, we only found these 4 adult males in the main study area, so we inferred they were the same individuals.

Attacks on adult females by adult males

A total of three attacks on adult females by adult males were observed during the study period. All attacking events occurred after four adult males immigrated into the group (Table 1). Two victims were females with unweaned infants.

The first attack was observed at 18:35 on 20 December, 2003. The group was resting in a large tree near the sleeping site. An adult male approached the adult female with the youngest infant. The female began run away as soon as she became aware of the male. The

male then chased the female. In this process, the infant was heard to scream loudly. Finally, the female escaped successfully from this attack and sat in a tree about 15 m away from the male. The female watched the male until he left. There was no body contact during this attack.

The second attack was observed on 21 December, 2003. At 18:25, the adult female with youngest infant first entered the sleeping cave on a cliff. At 18:30, an adult male moved quietly along the cliff to the sleeping cave. The adult female saw the approaching male and moved out quickly from the cave. Because the adult

female with the infant could not rapidly move along the cliff, the adult male approached and slapped her head. The attacking male then tried to take the infant from its mother, but was unsuccessful. Finally, the female jumped from the cliff to a high tree nearby, escaping this attack. In the process of attack, the infant was heard to scream loudly. At 18:35, the adult male approached another adult female with oldest infant, who was resting on the cliff ledge. The adult female detected the approaching male early and moved away quickly. Because it was dark out, the male did not follow the adult female. Finally, the adult females slept in another cave, about 20 m from where the adult males slept.

The third attack was observed on 22 December, 2003. At 15:15, all members of the group rested on a big tree of the hilltop. The adult males were approximately 5 meters from the adult females and their infants. An adult male suddenly launched an attack towards the female with the youngest infant. Afterwards, he directs similar attacks at 15:20, 15:32, 15:40. In the process of attack, the attacking male persistently chased the female, but no body contact occurred. Finally, the female climbed down the tree and hid in the bush.

DISCUSSION

Infanticidal episodes usually occur soon after group takeovers or the death of the resident male (van Schaik & Janson, 2000). Li (2000), for example, reported that three immature white-headed langurs (*Trachypithecus leucocephalus*) disappeared immediately after serious fighting between resident males and extra-group challengers. Zhao et al (2011) also reported that six infants disappeared after five group takeovers in white-headed langurs. Both suggested that infanticide may be the cause of the disappearance of the immature individuals. Although we did not witness a group takeover in this study, our observations may possibly be from the time just after the males drove the resident male away and took over the group. Sugiyama (1965) reported that in Hanuman langurs (*Semnopithecus entellus*), the attacking males repeatedly joined and left the group within several days after takeovers, consistent with the behaviors we observed among the François' langurs.

All attacks on adult females with infants occurred after the 4 adult males immigrated into the study group. An adult female with her unweaned infant disappeared two days after the third set of attacks. Since we did not find a dead body, we presume that the female and her infant dispersed. As a close phylogenetic relative of the white-headed langur, we can reasonably infer that the attacks by the males were infanticide attempts and may have been the cause the adult female and her infant's dispersal. Studies of other primates have noted similar cases of dispersal to avoid infanticide by males (e.g., *Cebus capucinus*, Jack & Fedigan, 2009; *Colobus vellerosus*, Teichroeb et al, 2009; *Propithecus edwardsi*, Morelli et al, 2009; *Tleucocephalus*, Zhao et al, 2011).

For the sexual selection hypothesis to be supported (Hrdy, 1979), the circumstances infanticide by males occurred requires the three conditions: (1) the infanticidal male is not related to the infant; (2) the mother goes into estrus more rapidly and conceives earlier after her infant is killed; and (3) the infanticidal male improves his chance of siring the subsequent infant. In this study, group composition changed during the study period, as 4 adult males left the group for 6-19 d at one time. Why the males left the group was unclear. All-females within the group appeared to be stable, and were moving, feeding, and resting as a cohesive group. Once the males joined the group, the adult females always kept a certain distance from them. The males also slept in a different cave or ledge on the cliff from the adult females and immatures at night (personal observation).

Collectively, the phenomena we observed during the course of this study suggest that the possibility the infants were related to the attacking males was low. However, this conclusion will still need to be confirmed by DNA analysis before we can draw any meaningful conclusions. Moreover, if the female with the youngest infant dispersed to avoid infanticide, the males would not have had mating access to her, therefore violating the sexual selection hypothesis. On the whole, these cases then do not completely verify the sexual selection hypothesis, but are neither inconsistent with it. Perhaps further observational studies can collect more data to clarify the relationship between infanticide and the sexual selection hypothesis.

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Seed caching and cache pilferage by three rodent species in a temperate forest in the Xiaoxinganling Mountains

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Abstract: Although differences in food-hoarding tactics both reflect a behavioral response to cache pilferage among rodent species and may help explain their coexistence, differentiation in cache pilfering abilities among sympatric rodents with different hoarding strategies is seldom addressed. We carried out semi-natural enclosure experiments to investigate seed hoarding tactics among three sympatric rodent species (*Tamias sibiricus*, *Apodemus peninsulae* and *Clethrionomys rufocanus*) and the relationship of their pilfering abilities at the inter- and intraspecific levels. Our results showed that *T. sibiricus* exhibited a relatively stronger pilfering ability than *A. peninsulae* and *C. rufocanus*, as indicated by its higher recovery rate of artificial caches. Meanwhile *A. peninsulae* showed a medium pilfering ability and *C. rufocanus* displayed the lowest ability. We also noted that both cache size and cache depth significantly affected cache recovery in all three species. *T. sibiricus* scatter-hoarded more seeds than it larder-hoarded, *A. peninsulae* larder-hoarded more than scatter-hoarded, and *C. rufocanus* acted as a pure larder-hoarder. In *T. sibiricus*, individuals with lower pilfering abilities tended to scatter hoard seeds, indicating an intraspecific variation in hoarding propensity. Collectively, these results indicated that sympatric rodent species seem to deploy different food hoarding tactics that allow their coexistence in the temperate forests, suggesting a strong connection between hoarding strategy and pilfering ability.

Keywords: Caching pilfering; Hoarding strategy; Sympatric rodents; Coexistence

How similar species coexist on similar resources has been a longstanding interest to community ecologists (Randall, 1993; Schoener, 1986). At present, the factors that influence species coexistence are poorly understood, especially among the terrestrial food-hoarding rodents. Several rodent species which are superficially alike in appearance and habit seem to coexist despite relying on the same limited food sources (Chang & Zhang, 2011; Kotler & Brown, 1988). Potentially, behavioral differentiations at the intraspecific or interspecific level may function as a mechanism to facilitate this kind of coexistence (Ben-Natan et al, 2004; Perri & Randall, 1999).

Food hoarding is an evolutionary adaptation that allows animals to manipulate food availability both spatially and temporally (Vander Wall, 1990), thereby increasing chances of survival and reproductive success during periods of food shortage (Post et al, 2006). Jenkins & Breck (1998) and Price et al (2000) predicted that differences in food hoarding strategies among rodent species may allow or facilitate their coexistence. Animals usually store food in two different ways in terms of the

spatial distribution of the stored items: larder-hoarders store large quantities of food in central locations, e.g., burrows, while scatter-hoarders distribute small caches of food items in numerous shallow pits within their home range (Jenkins & Breck, 1998; Vander Wall, 1990). Each strategy is not without risks. Larders, for instance, are vulnerable to catastrophic loss if the individual fails to defend the resources from intraspecific or interspecific pilferers (Dally et al, 2006; Stapanian & Smith, 1978; Vander Wall & Jenkins, 2003). In theory, scatter-hoarding is supposed to reduce this risk (Leaver, 2004; Stapanian & Smith, 1978, 1984; Vander Wall, 1990), but it involves a greater input of energy to recover the scattered caches (Male & Smulders, 2007; Stapanian & Smith, 1978; Vander Wall, 1990, 2000). Similarly, much like larders, scatter-hoards are pilfered by sympatric competitors (Leaver, 2004; Leaver & Daly, 2001; Preston

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& Jacobs, 2001; Vander Wall & Jenkins, 2003). Therefore, pilfering is common and regarded as an alternative foraging strategy adopted by rodents and birds (Dally *et al.*, 2006; Iyengar, 2008; Vander Wall & Jenkins, 2003) and may greatly influence on the respective hoarding strategies of different animals.

Based on the assumption that pilfering can be considered an alternative foraging strategy that then impacts hoarding strategies, it seems likely that species would diverge in their abilities to exploit different food hoarding tactics in order to coexist. Unfortunately, food hoarding tactics of coexisted species are seldom studied (but see Leaver & Daly, 2001; Price & Mittler, 2003; Vander Wall *et al.*, 2009). Here, we investigated the pilfering abilities and hoarding strategies of three small rodent species coexisting in a temperate forest (*Tamias sibiricus*, *Apodemus peninsulae* and *Clethrionomys rufocanus*). The three rodent species rely on seasonally abundant seeds from local tree species *Pinus koraiensis*, *Corylus mandshurica*, *Corylus heterophylla*, and *Quercus mongolica* as their main food supply (Yi & Zhang, 2008; Yi *et al.*, 2011a, b). *A. peninsulae*, a nocturnal species, mainly larder-hoard but seldom scatter-hoard seeds. Another nocturnal species *C. rufocanus* acts as a pure larder-hoarder, though their food mainly consists of roots and bark (Yi, personal observation, data not published). Meanwhile, the Siberian chipmunks, *T. sibiricus*, are a diurnal rodent species and mainly scatter-hoard seeds (Yang *et al.*, 2012a, b; Yi *et al.*, 2011a, b). Our aim was to test two hypotheses: 1) Coexisting species relying on the same limited seed sources may display different hoarding strategies; 2) Unlike larder-hoarders, scatter-hoarders make small caches of food items throughout their home range, and are expected to rely more on spatial memory and olfaction to recover their scattered caches, and accordingly rodent species performing different hoarding strategies should exhibit different pilfering abilities.

MATERIALS AND METHODS

Study site

The study was conducted in the semi-natural enclosures in northeastern China at the Dongfanghong Forestry Center (average elevation 750 m, located at N46°50'–46°59', E128°57'–129°17') in Dailing district, Yichun city, Heilongjiang Province. The climate of the experimental site is dominated by northern temperate zonal monsoons with long, severe winters and short summers. The annual average air temperature is 14 °C with a maximum of 37 °C and minimum of –40 °C. Average annual precipitation averages 660 mm, 80% of which falls in the short growing season from May to September (Yi & Zhang, 2008). The study site was located in a mixed secondary broad-leaved and mixed

conifer forest. In the experimental region, common tree species include *Betula platyphlla*, *Juglans mandshurica*, *Quercus mongolica*, *Pinus koraiensis*, *Fraxinus mandshurica*, *Phellodendron amurese*, *Acer mono* and *Tilia amurensis*; beneath the tree canopy, common shrubs are *Corylus mandshurica*, *Corylus heterophylla*, *Fructus schisandrae* and *Acanthopanax senticosus*.

Establishment of enclosures

We conducted experiments in separate 10 m×10 m semi-natural enclosures that had been previously established in an open and level area. The enclosures were built using 3 m tall bricks placed in the ground, with about 2.5 m high above ground and 0.5 m below the soil surface. The walls of the enclosures were smoothed to prevent escape of small rodents. To prevent predators from entering the enclosures from outside, the enclosures were covered with plastic nets on the top. To allow animals to rest and drink freely, an artificial nest area was constructed of bricks (H×W×L: 20 cm×15 cm×30 cm) in one corner of the enclosure and provisioned with a water bowl. A seed station of 1 m² was established at the center of each enclosure.

Capture of rodent species

Steel frame live traps (H×W×L: 9 cm×10 cm×25 cm), baited with peanuts and carrot, were placed in the forest at 5 m intervals along four transects at 09:00. We checked the live traps every 3 h to ensure the safety of the captured rodents. The animals captured in each visit were transported by vehicle to the laboratory housing room within 30 min. All traps were then taken back at 18:00 and re-placed in the forest the following day. Trapping stopped on days with bad weather, i.e., heavy raining. Rodents transported to the laboratory were kept individually in steel frame cages (H×W×L: 40 cm×50 cm×90 cm) at a natural temperature (15–25 °C) and photoperiod (14 h of light). All specimens were provided with carrots, peanuts, tree seeds and water *ad libitum*. No animal died during the trapping and laboratory feeding processes. In total, we collected 23 specimens including *T. sibiricus* (*n*=7), *A. peninsulae* (*n*=9) and *C. rufocanus* (*n*=7) with respective mature body masses of 104.80±9.25 g, 26.83±5.64 g and 33.38±3.57 g (mean±SD).

Seed hoarding tactics

We used Korean pine (*P. koraiensis*) seeds because they are seasonally abundant and the most important food sources of all three rodent species. *P. koraiensis* produces medium-sized seeds (L×W: 1.60 cm×1.11 cm; fresh weight: 0.73±0.05 g) that are characterized by low tannin and high caloric value (Yang *et al.*, 2012a). Seeds were labeled with plastic tags according to the previously published method (Yi & Zhang, 2008) with minor

modifications. A hole 0.3 mm in diameter was drilled through the husk, far from the embryo of each seed, without damaging the cotyledon and the embryo. A flexible plastic tag (2.5 cm×3.5 cm, <0.3 g) was tied through the hole in each seed using a thin steel thread, 10 cm long. Seed placement was based on the daily consumption of the three rodent species. For *T. sibiricus*, 30 intact tagged Korean pine seeds were placed at the seed station in each enclosure only once at 07:00 and seed fates were checked at 18:00. For *A. peninsulae* and *C. rufocanus*, 20 seeds were supplied at 18:00 and seed fates were checked in the next morning (08:00). Seed fates were defined using the following abbreviations: intact in situ (IS), eaten in situ (EIS), eaten after removal (EAR), intact after removal (on surface) (IAR), scatter-hoarded (SC) and larder-hoarded (LC). Larder-hoarded seeds were defined as those carried into the artificial nests or belowground nests, while scatter-hoarded seeds referred to those buried in small pits on the surface of ground. In total, we tested 14 *T. sibiricus* (6♀, 8♂), 8 *A. peninsulae* (4♀, 4♂) and 7 *C. rufocanus* (4♀, 3♂) individuals in hoarding trials. Animals were given the opportunity to bury seeds in the enclosures.

Cache pilfering

To test the pilfering abilities of the three rodent species, the same combination of artificial caches was provided. We established 9 categories of caches in each semi-natural enclosure that differed both in burial depth (1 cm, 2 cm or 5 cm) and the number of Korean pine seeds (1, 2 or 5): 1 seed at 1 cm, 2 cm, and 5 cm; 2 seeds at 1 cm, 2 cm, and 5 cm; and 5 seeds at 1 cm, 2 cm, and 5 cm. Each category was replicated six times, making for a total of 54 caches with 144 seeds in each enclosure. Caches of the same category were spaced more than 2 m apart, and caches of different categories were more than 80 cm apart randomly and evenly distributed in each enclosure. This array of cache categories is supposed to represent actual caches in the field according to burial depth and cache size (Yi et al, 2008). To minimize the influence of artificial clues, each individual of the three rodent species was introduced into the enclosures three days after cache preparation. In the first trial round, 15 *T. sibiricus* (7♀, 8♂) were simultaneously put into 15 enclosures for daytime testing. In the second round, 8 *A. peninsulae* (4♀, 4♂) and 6 *C. rufocanus* (3♀, 3♂) were introduced at night. We provided water but no food other than the experimental seeds. Two days later, we surveyed the enclosures to determine which caches were removed. Removed caches were termed as those that one or more seeds were excavated by rodents.

To further test whether there was an intraspecific variation in pilfering ability of *T. sibiricus*, we randomly selected 32 *T. sibiricus* (17♀, 15♂) and provided them with 30 pine seeds at the seed station of each enclosure every morning. In the afternoon, all seeds and seed

debris were cleaned and the proportion of cached seeds was recorded for four consecutive days. After the four days, the 10 individuals (5♀, 5♂) of both the highest ($\geq 30\%$) and lowest ($\leq 3\%$) caching propensity (termed as the proportion of cached seeds) were selected for further analysis via pilfering trials. We established three categories of caches in each enclosure: 1 seed at 1 cm, 2 cm, and 5 cm deep. Each type of cache was replicated 30 times, for a total of 90 caches (90 seeds) in each enclosure. One individual of each group was introduced in each enclosure in the morning. The number of caches that were removed was checked two days later.

Following the experiments, all animals were released to the site of their initial capture. The trapping and housing of animals were permitted by the Henan University of Science and Technology (No. HAUST015).

Data analysis

We used SPSS 16.0 (SPSS inc., Chicago, USA) for data analysis. Three-way ANOVA was used to see the effects of rodent species, cache size, and cache depth on the removal rates of artificially cached seeds. Differences in the number of scatter-hoarded seeds selected by the chipmunks with different caching propensity were detected using general linear models (GLM). Difference in the proportion of scatter- and larder-hoarded seeds by the three rodent species was also tested using GLM. Data were arc-sin transformed before analyses.

RESULTS

Our results indicated that *A. peninsulae* both scatter-hoarded and larder-hoarded Korean pine seeds, with more seeds being larder-hoarded ($F_{1,14}=13.39$, $df=1$, $P=0.003$). *T. sibiricus*, meanwhile, scatter-hoarded more seeds than it larder-hoarded ($F_{1,26}=24.69$, $df=1$, $P<0.001$). *C. rufocanus* did not scatter-hoard at all; instead, it acted as a pure larder-hoarder (Figure 1).

The three rodent species displayed different abilities

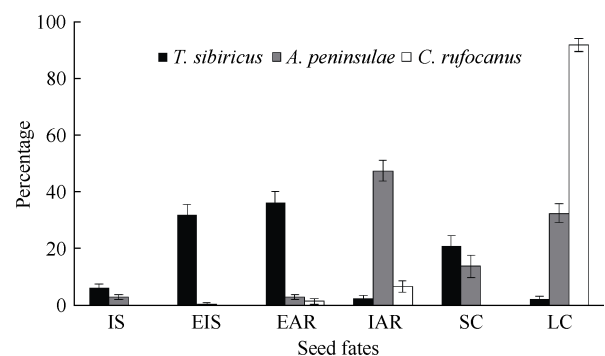


Figure 1 Seed fates manipulated by three sympatric rodent species in semi-natural enclosures

Abbreviations: IS: intact in situ; EIS: eaten in situ; EAR: eaten after removal; IAR: intact after removal; SC: scatter-hoarded; and LC: larder-hoarded. Data are expressed as mean±SE.

to pilfer Korean pine seeds from artificial caches ($F_{2,8}=178.83$, $P<0.001$). *T. sibiricus* exhibited a stronger pilfering ability than *A. peninsulae* and *C. rufocanus* ($P<0.05$). *A. peninsulae* excavated more caches than *C. rufocanus* ($P<0.001$) (Figure 2). Pilfering abilities of the three rodent species decreased dramatically with the

depth of artificial caches ($F_{2,8}=45.289$, $P<0.001$). Seeds at 5 cm deep caches were less likely to be removed than those at 1 and 2 cm ($P<0.05$). Cache size also was found to influence removal rates ($F_{2,8}=3.67$, $P=0.027$), caches containing 5 seeds were more likely to be pilfered than those with 1 or 2 seeds ($P<0.05$) (Figure 2).

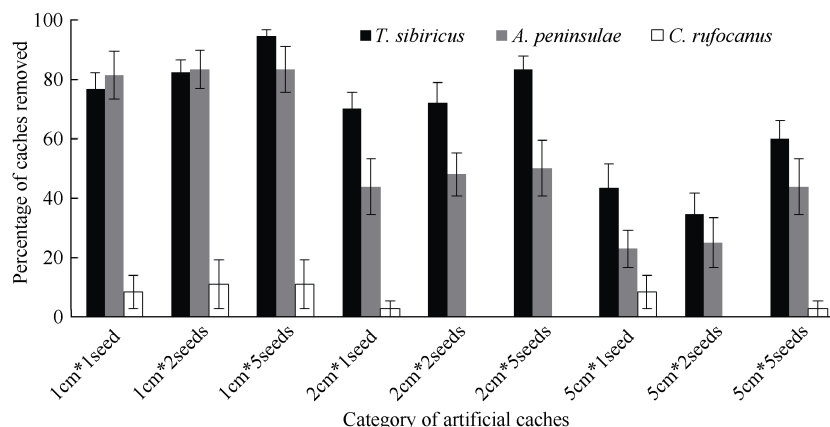


Figure 2 Number of caches excavated by three sympatric rodent species at nine combinations of cache sizes in semi-natural enclosures

Data are expressed as mean \pm SE. Error between replicate single individuals of each species was tested individually.

Table 1 Cache recovery rates in response to variations in cache size, cache depth, and rodent species

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	861.175 ^a	26	33.122	21.036	0.000
	1 456.732	1	1456.732	925.181	0.000
Cache size	11.558	2	5.779	3.670	0.027
Cache depth	142.620	2	71.310	45.289	0.000
Rodent species	563.149	2	281.575	178.830	0.000
Cache size \times Cache depth	4.203	4	1.051	0.667	0.615
Cache size \times Rodent species	9.457	4	2.364	1.502	0.202
Cache depth \times Rodent species	56.951	4	14.238	9.042	0.000
Cache size \times Cache depth \times Rodent species	3.243	8	0.405	0.257	0.979
Error	368.442	234	1.575		
Total	3 694.000	261			
Corrected Total	1 229.617	260			

^a R-Squared=0.700 (Adjusted R-Squared=0.667).

We found an apparent intraspecific differentiation in caching propensity of *T. sibiricus* (Figure 3). 19/32 individuals actively scatter-hoarded Korean pine seeds, whereas 13 either seldom or did not scatter-hoarded at all ($F=37.34$, $df=1$, $P<0.001$) (Figure 3). Interestingly, *T. sibiricus* individuals that actively cached exhibited a low ability to pilfer seeds from artificial caches. However, chipmunks that seldom cached seeds removed

significantly more seeds from artificial caches ($F=13.92$, $df=1$, $P<0.001$) (Figure 3). Cache depth did not alter the removal rates by chipmunks ($F=1.102$, $df=2$, $P=0.339$). We also found that individuals of *T. sibiricus* that did not cache tended to immediately eat the seeds they recovered from artificial caches ($F=16.02$, $df=1$, $P<0.001$) (Figure 3).

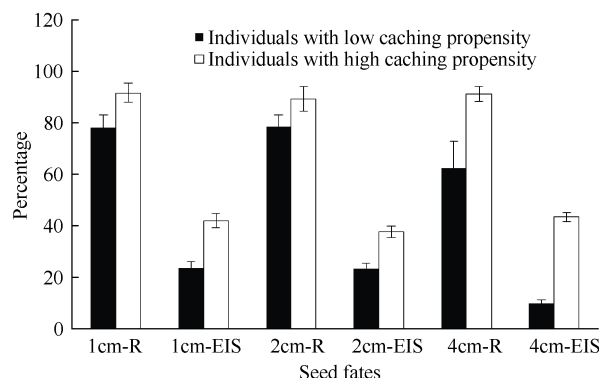


Figure 3 Number of caches excavated by active and inactive caching Siberian chipmunks at three cache depths in semi-natural enclosures

Abbreviations: R: removed; EIS: eaten *in situ*. Data are expressed as mean \pm SE.

DISCUSSION

Differentiation in food hoarding strategies among coexisting rodent species has been found in other ecosystems (Price & Mittler, 2003; Lu & Zhang, 2008;

Vander Wall et al, 2009; Chang & Zhang, 2011; Huang et al, 2011) and is considered as an adaptation to coexistence (Jenkins & Breck, 1998; Price et al, 2000; Vander Wall, 2000; Leaver & Daly, 2001). In the present study, we found a significant interspecific variation in food hoarding strategies among individuals of all three sympatric rodent species. *T. sibiricus* and *A. peninsulae* exhibited both scatter-hoarding and larder-hoarding, while *C. rufocanus* completely larder-hoarded. *T. sibiricus* and *A. peninsulae* mainly rely on seeds of several local tree species and have an overlap of food niches (Yi & Zhang, 2008), and show a similar hoarding strategy foraging on the other local seed species (unpublished data). Our results further support the idea this kind of differentiation in food hoarding strategies may allow or facilitate the coexistence of sympatric rodent species by reallocating of food resources along an axis of resource heterogeneity (Kotler & Brown, 1988).

As earlier studies have noted, both larder- and scatter-hoarding suffer some disadvantages, such as substantial cache loss and increased costs of food handling (i.e., harvested seeds are transported and cached, then dug up and eaten) (Vander Wall, 1990). Accordingly, the evolution of the mechanism underlying the two hoarding tactics remains uncertain. Andersson & Krebs (1978) suggested that hoarding behaviors evolve when the caching individual is more likely than any other to gain access to their caches. However, more recent studies have noted that rodents significantly increase the intensity of scatter-hoarding but not larder-hoarding when faced with complete cache loss of scatter-hoarded seeds (Huang et al, 2011, but see Preston & Jacobs, 2001). Only a few studies have delved further and investigated the connected behaviors involved in interspecific differences of pilfering abilities (Leaver & Daly, 2001; Thayer & Vander Wall, 2005). Our results showed that rodent species with strong pilfering ability (*T. sibiricus*) tended to rely on scatter-hoarding seeds rather than larder-hoarding. However, rodent species with extremely weak pilfering ability (*C. rufocanus*) completely larder-hoarded food items in their burrows or nests, likely avoiding pilferage by *T. sibiricus* and *A. peninsulae*. Given this finding, it is plausible that the relatively higher pilfering ability of *T. sibiricus* and *A. peninsulae* constrain *C. rufocanus* in to larder-hoarding seeds. To our knowledge, this may be one of only a few studies to suggest a relationship between hoarding strategy and pilfering ability in small rodents (Vander Wall et al, 2009). We further found that the medium

pilfering ability of *A. peninsulae* may explain its dependence on both scatter-hoarding and larder-hoarding because taking a solely scatter-hoarding or larder-hoarding strategy would subject it to substantial losses of hoarded food by *T. sibiricus* (caches) or *C. rufocanus* (larders). Subsequently, adopting scatter-hoarding combined with larder-hoarding may be a novel way for *A. peninsulae* to cope with these pilfering risks.

Current theories state that food-hoarding tactics can be adaptive if a hoarder has a higher priority than any other animal to retrieve its own caches (Vander Wall & Jenkins, 2003). Although larger species are better able to physically defend a larder (Preston & Jacobs, 2001), *T. sibiricus*, possessing the highest body mass in our comparison, performed scatter-hoarding rather than larder-hoarding. This finding suggests that body size (and by extension, defensive ability) is not always a valid explanation of the selection of scatter-hoarding versus larder-hoarding among sympatric rodent species (Dally et al, 2006).

Cache pilfering occurs not only between different species (Burnell & Tomback, 1985), but also between within them (Heinrich & Pepper, 1998), therefore leading to intraspecific variations of hoarding tactics. Our results concurred with this assessment, as we noted an apparent intraspecific differentiation in hoarding strategies among *T. sibiricus*. Individuals of *T. sibiricus* with lower pilfering ability tended to scatter-hoard more seeds than those who possessed a higher pilfering ability, implying that non-hoarding individuals are common in rodent populations. Individuals of *T. sibiricus* with higher pilfering ability are expected to rely on food resources pilfered from caches of conspecifics or interspecifics. Conversely, individuals with lower pilfering ability tend to perform scatter hoarding tactics and recover their caches mainly through spatial memory. Therefore, scatter hoarding is predicted to be an alternative strategy for individuals with lower pilfering ability to prevent cache losses (MacDonald, 1997; Preston & Jacobs, 2001; Gálvez et al, 2009). In summary, our results indicate that sympatric rodent species seem to deploy different food hoarding tactics that allow their coexistence in the temperate forests, suggesting a strong connection between hoarding strategy and pilfering ability.

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Seasonal variations in the energy budget of Elliot's pheasant (*Syrmaticus ellioti*) in cage

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Abstract: This study aimed to discuss the energy budget of Elliot's pheasant *Syrmaticus ellioti* in different seasons, with life and health, good growth and normal digestion of Elliot's pheasant as the tested objects. The energy budget of Elliot's pheasant was measured by daily collection of the trial pheasants' excrement in the biological garden of Guangxi Normal University from March 2011 to February 2012. The results showed that the gross energy consumption, metabolic energy and excrement energy varied by season, increasing as temperature decreased. There was significant difference in gross energy consumption, metabolic energy, excrement energy between adults and nonages. There was also a trend that food digestibility of pheasants increases as temperature increases. In the same season, the food digestibility of adults was better than that of nonages. Throughout spring, summer, autumn and winter, the metabolic energy of 4-year adults were 305.77 ± 13.40 kJ/d, 263.67 ± 11.89 kJ/d, 357.23 ± 25.49 kJ/d and 403.12 ± 24.91 kJ/d, respectively, and the nonages were 284.86 ± 17.22 kJ/d, 284.66 ± 15.16 kJ/d, 402.26 ± 31.46 kJ/d and 420.30 ± 31.98 kJ/d, respectively. The minimum metabolic energies were 247.65 ± 21.81 g, 265.86 ± 26.53 g, respectively for each group, detected between 4-year adults and 1-year nonages. Further study is needed to determine whether 29.6 °C is the optimal temperature for the Elliot's pheasant.

Keywords: Elliot's pheasants; Energy budget; Seasonal variations

Elliot's pheasant *Syrmaticus ellioti* is a vulnerable species peculiar to China (Baillie et al, 2004), mainly distributed across the eastern hilly sub-region in central China, including Zhejiang, Anhui, Fujian, Jiangxi, Hubei, Hunan, Guangdong, and Guangxi provinces (Delacour, 1977; Ding & Zhu, 1989; Li, 1996; Ding, 1998). Mainly inhabiting rugged mountains and jungle of valleys at an altitude of 200–1 500 m, more commonly in mixed conifer-broad leaved forests, Elliot's pheasant can also be found in dense bamboo and forest understory. Elliot's pheasant is omnivorous, mainly eating plant leaves, stems, buds, flowers, fruits, seeds and other crops, but also insects and other animals (John et al, 2000). Due to chronic deforestation, burning and vegetation reclamation, agricultural encroachment and the stress of hunting among other factors, Elliot's pheasant is approaching of the serious loss, fragmentation and degeneration of its habitat (Ding et al, 2000). According to the survey on typical habitat of Elliot's

pheasant in Kaihua County, Zhejiang in 1984, the population density of Elliot's pheasant was $3.5/\text{km}^2$ in summer and $6.9/\text{km}^2$ in winter (Li, 1985).

Since the 1980s, domestic researchers have studied the field ecology of the form, distribution, living habits, activity regularity, breeding habits, habitat types of Elliot's pheasant, and learned about the wild pheasant populations (Li, 1985; Long, 1985; Ding & Zhu, 1988). Since the 1990s, the "3S" technologies were used widely used for the study of the pheasant's inhabiting features, selection mechanism as well as habitat type and feature (Ding et al, 1996, 2001; Shi & Zheng, 1997), habitat vegetation fragmentation (Ding et al, 2000) and activity

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area (Cai *et al.*, 2007; Xu *et al.*, 2007). Researchers have likewise studied more recently the genetic diversity, genetic structure and genetic flow on the basis of mitochondria DNA in Elliot's pheasant (Jiang *et al.*, 2005a, 2005b, 2007). However, despite recent progress, the previous studies seldom deal with the energy budget and feed intake of Elliot's pheasant (Luo *et al.*, 2007, 2011), a significant feature for studying the variation of energy ecology of Phasianidae that live in the subtropical shrub ecosystems.

The birds' capabilities of gaining energy depend on food digestibility and energy acquisition from food in limited time, or more succinctly, only the available energy in an environment is turned into chemical energy before it can be used (Lu, 1991). Food is the basis adaptation to environmental surroundings and maintaining their lives, Elliot's pheasant can get the energy from the everyday food, helping to keep their normal day to day activities in check and build their own bodies. Studying the energy intake of Elliot's pheasant in cage will greatly contribute to grasping their food digestibility, and inferring the energy demand of maintaining wild Elliot's pheasant, while also laying the basis of the population energy ecology and the environmental carrying capacity needed for successful reintroduction of Elliot's pheasant. At the same time, this study also provides a scientific foundation for more adequate management of Elliot's pheasant in captivity. Between March 2011 and February 2012, we researched the different seasonal energy intake of Elliot's pheasant at different age in the Breeding Base of Endangered Pheasants, Guangxi Province - the Biological Garden of Guangxi Normal University.

MATERIALS AND METHODS

Animals

According to the mating system of the wild Elliot's pheasant (1 male, 2 or 3 females), we selected 12 Elliot's pheasants of different ages, that were alive and healthy, good growth and normal digestion from the Biological Garden of Guangxi Normal University in March 2011, 6 of them were 4-year old (2 males, 4 females), and once bred. The number of other group of trial pheasant were also 6 (2 males, 4 females), which be hatched in May 2010. Since Elliot's pheasant have strong stress, in order to make the trial pheasants adapt to the environment of separate feeding, the pre-experiments had to be done before the post-experiment. Cages in which the trial pheasants lived were disinfected before every experiment preventing the specimens from being sick. There were plenty of favorite foods for Elliot's pheasants.

Methods

The energy balance of Elliot's pheasant was

measured by the daily collection of the trial pheasants' excrement (Zhang *et al.*, 2004). During the experiment, the birds were kept alone in a cage that consisted of an inner and outer room. The inner room (1.9 m×2.8 m) was used for feeding and rest, with one food manger hanging on the wall about 10 cm off the ground. The outer room (3.5 m×2.8 m) was used for movement, and the walls and nets of outer room were 1.25 m and 0.7 m height, respectively. The cage grounds were covered with double films. There were a variety of flowers and trees around the cage and the environment was very quiet. In order to make the trial pheasants adapt separate feeding, the pre-experiment was 8 days, then, the post-experiment was 6 days. All the Elliot's pheasants were weighed hungrily with electronic scales before and after each experiment.

The foods of Elliot's pheasant consisted of dried corns and compound forages, which were put in the food manger, and cabbages were hung on the wall with a thin wire. The trial pheasants were fed 2 times, at 08:00 and 13:00 each day, and their excrement and urine acid were collected 3 times with the stainless steel spoons and plastic syringes, respectively, at 08:00, 13:00 and 18:00 each day. Surplus baits were collected at 18:00 each day. The food intake of every bird was weighed and calculated with the Pallet scales each day. All the collective excreta samples were mixed, signed and placed in the Petri dish, then, kept in the vacuum oven at a constant heat of 65 °C for 3–4 days. The cabbages were also hung around the cage to correct the evaporation of cage's cabbages. According to the actual situation of Guilin's climate, the experiment times can be divided into spring (March–May), summer (June–August), autumn (September–November) and winter (December–February), and the temperatures of spring, summer, autumn and winter were 20.3, 28.0, 18.6 and 8.1 °C, respectively. Energy metabolism experiments were made every month under the natural temperature. Lastly, we took the average temperature between maximum and minimum as the day temperature and the average of 6 days formal experiments as trial temperature. From January. to December in the whole year, there were 12 groups' of temperatures were measured, which were 7.2, 5.0, 14.5, 22.0, 24.5, 26.5, 28.0, 29.6, 25.2, 18.5, 12.0 and 9.0 °C.

Many studies have shown there were no sex difference between food intake and excreta, and accordingly we did not take the sex difference into consideration during this experiment (Kendengh, 1970; Wang *et al.*, 1996).

The food digestibility (%) was calculated by using the following formula: $D = (E_i - E_e) / E_i$, in which D is food digestibility (%), E_i is gross energy intake, E_e is excrement energy. The calorific values of corns, compound forages and Chinese cabbages were measured

with GR-3500 oxygen bomb calorimeter (Changsha Instruments).

Body weight

The body weight of trial pheasants were measured with the electronic scales precise to 0.1 g. After Elliot's pheasant nestlings were hatched by machine in May 2010 and their feather dried, their body mass were measured and regarded as 0d index; 1–30 days old nestlings body mass was recorded daily; 31± days body masses were recorded once every 2 days, 46± days old were recorded once every 3 days, 55± days old were recorded once every 5 days, 70± days old were recorded once every 10 days, until nestlings reached 100 days old. The body mass of the 4-year old adults were measured monthly, before and after the formal experiment, when the specimens were hungry.

Data analysis

Data were analyzed by using the SPSS11.5 statistical package. All results were calculated by one-way ANOVA and expressed as mean±SE. The differences of the same years' groups among different seasons were analyzed by post hoc multiple comparisons ANOVA, and the differences of the same seasons among different years' groups were analyzed using paired *t*-Test, with ($P<0.05$) taken as statistically significant.

RESULTS

Gross energy intake, metabolic energy and food digestibility

Season gross intake, metabolic energy and food digestibility of Elliot's pheasant were analyzed by using post hoc multiple comparisons ANOVA and paired *t*-Test (Table 1, Figure 1, Table 2, Figure 2), respectively. The results showed that there were significant differences among the gross energy intake of Elliot's pheasant (4-year adults: $F=9.470$, $P=0.000<0.05$, $n=72$; 1-years nonages: $F=9.427$, $P=0.000<0.05$, $n=72$), and that the gross energy intake and metabolic energy were highest in winter and lowest in summer. In the same season, the gross energy intake of 1-year nonages was greater than for 4-year adults except spring ($t=2.472$, $df=71$, $P=0.016<0.05$, Table 1, Table 2). Likewise the metabolic energy of 1-years nonages was also larger than 4-year adults except for during spring ($t=1.940$, $df=71$, $P=0.046<0.05$, Table 1, Table 2). The average food digestibility (%) of 4-year adults and 1-years nonages were $(85.44\pm0.54)\%$ and $(84.14\pm0.55)\%$, respectively, indicating that the food digestibility (%) of 4-year adults was better than that of 1-years nonages ($t=6.159$, $df=71$, $P=0.000<0.05$). With the temperature increase, the gross energy intake, metabolic energy and food digestibility of the different age groups of Elliot's pheasants lowered all 12 months of the year (Table 3, Table 4). The 29.6 °C in August was the highest temperature and the metabolic energy was the smallest, while the 5.0 °C of February was the lowest temperature and the metabolic energy was largest over the entire year.

Excreta, excrement energy and excrement calorific value

The excreta, excrement energy and excrement

Table 1 Seasonal energy intake of 4-year adults

	Spring	Summer	Autumn	Winter	<i>n</i>	<i>F</i>	<i>P</i>
Food intake (g/d)	22.7±1.3 ^a	19.1±1.3 ^b	26.4±3.4 ^c	28.8±2.3 ^c	72	17.675	0.000
Gross energy intake (kJ/d)	362.39±14.81 ^a	305.36±13.64 ^a	429.09±29.80 ^b	462.11±27.74 ^c	72	9.470	0.000
Excreta (g/d)	4.31±0.62 ^a	3.32±0.60 ^b	5.36±0.69 ^c	4.49±0.41 ^a	72	27.279	0.000
Excrement energy (kJ/d)	56.45±2.05 ^a	41.69±2.13 ^b	71.86±4.45 ^c	58.77±2.85 ^a	72	16.673	0.000
Metabolic energy (kJ/d)	305.77±13.40 ^a	263.67±11.89 ^a	357.23±25.49 ^a	403.12±24.91 ^b	72	9.287	0.000
Food digestibility (%)	84.13±1.78 ^a	86.92±0.73 ^b	83.34±0.56 ^c	87.09±0.75 ^b	72	49.258	0.000

Data of Table 1 were analyzed by using post hoc multiple comparisons ANOVA. Data with different lowercase letters in a row are regarded as significant or very significant differences ($P<0.05$).

Table 2 Seasonal energy intake of 1-years nonages

	Spring	Summer	Autumn	Winter	<i>n</i>	<i>F</i>	<i>P</i>
Food intake (g/d)	24.4±2.1 ^a	20.9±1.9 ^a	30.3±3.5 ^b	30.6±2.7 ^b	72	9.943	0.000
Gross energy intake (kJ/d)	337.86±19.02 ^a	335.60±17.99 ^a	486.14±36.50 ^b	492.79±35.85 ^b	72	9.427	0.000
Excreta (g/d)	4.21±0.12 ^a	4.11±0.28 ^a	6.16±0.12 ^b	5.69±0.46 ^b	72	23.562	0.000
Excrement energy (kJ/d)	52.97±2.04 ^a	50.94±2.87 ^a	82.81±5.40 ^b	72.49±4.07 ^c	72	37.491	0.000
Metabolic energy (kJ/d)	284.86±17.22 ^a	284.66±15.16 ^a	402.26±31.46 ^b	420.30±31.98 ^b	72	8.520	0.000
Food digestibility (%)	83.80±1.34 ^a	84.79±0.43 ^b	82.76±1.63 ^c	84.83±0.43 ^b	72	3.563	0.000

The data of Table 2 were analyzed by using post hoc multiple comparisons ANOVA. Data with different lowercases letters in a row is regarded as significant or very significant differences ($P<0.05$).

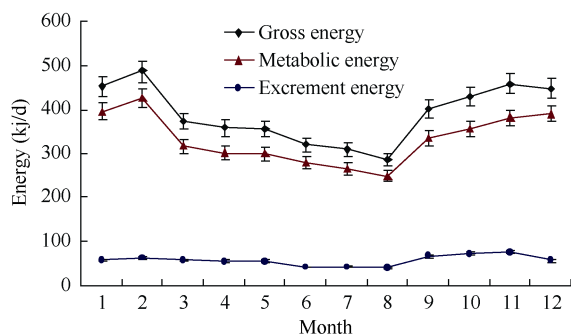


Figure 1 Monthly change in the energy intake of 4-year adults

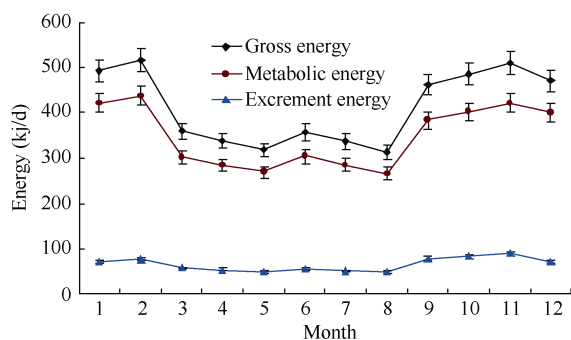


Figure 2 Monthly change in the energy intake of 1-year nonages

Table 3 Monthly energy intake of 4-year adults

Month	Temperature (°C)	Cross energy (kJ/d)	Metabolic energy (kJ/d)	Excrement energy (kJ/d)
1	7.2	452.74±49.04 ^a	395.15±44.17 ^a	57.59±4.94 ^a
2	5.0	486.54±53.34 ^a	424.60±47.82 ^a	61.94±5.58 ^a
3	14.5	373.88±26.82 ^b	315.32±24.29 ^b	58.56±2.87 ^a
4	22.0	357.80±31.21 ^b	302.29±28.05 ^b	55.51±3.57 ^a
5	24.5	355.50±22.42 ^b	299.70±26.64 ^b	55.30±4.54 ^a
6	26.5	323.59±23.93 ^b	280.99±20.68 ^b	42.60±3.29 ^b
7	28.0	308.69±25.03 ^b	264.86±22.23 ^b	43.84±4.17 ^b
8	29.6	287.13±25.96 ^b	247.65±21.81 ^b	39.48±4.47 ^b
9	25.2	401.85±55.15 ^a	334.82±46.90 ^a	67.03±8.27 ^a
10	18.5	429.08±54.54 ^a	356.34±46.92 ^a	72.73±8.26 ^a
11	12.0	457.04±52.69 ^a	380.70±44.97 ^a	76.34±7.75 ^a
12	9.0	446.39±48.67 ^a	389.61±43.77 ^a	56.78±4.91 ^a

The data of Table 3 were analyzed by using post hoc multiple comparisons ANOVA. Data with different lowercases letters in a row is regarded as significant or very significant differences ($P<0.05$).

caloric values of Elliot's pheasant were analyzed using post hoc multiple comparisons ANOVA and paired t -Test. The results indicated there were significant differences for the excreta (4-year adults: $F=72.279$, $P=0.000(0.05$, $n=72$; 1-years nonages: $F=23.562$, $P=0.000<0.05$, $n=72$), and excrement energy (4-year adults: $F=16.673$, $P=0.000<0.05$, $n=72$; 1-year nonages: $F=97.491$, $P=0.000<0.05$, $n=72$). Of Elliot's pheasant in the same age,

Table 4 Monthly energy intake of 1-year nonages

Month	Temperature (°C)	Cross energy (kJ/d)	Metabolic energy (kJ/d)	Excrement energy (kJ/d)
1	7.2	492.78±65.49 ^a	421.96±59.76 ^a	70.82±6.27 ^a
2	5.0	514.90±62.83 ^a	438.27±55.58 ^a	76.63±7.42 ^a
3	14.5	358.52±34.41 ^b	301.27±31.42 ^b	57.25±3.39 ^a
4	22.0	337.85±34.92 ^b	284.08±31.46 ^b	53.78±3.77 ^b
5	24.5	317.20±33.39 ^b	269.25±30.77 ^b	47.95±2.79 ^b
6	26.5	357.49±32.64 ^b	303.47±27.45 ^b	54.03±5.21 ^b
7	28.0	335.60±32.17 ^b	284.66±27.33 ^b	50.94±5.03 ^b
8	29.6	313.71±31.77 ^b	265.86±26.53 ^b	47.85±5.24 ^b
9	25.2	462.24±66.13 ^a	384.45±57.09 ^a	77.79±9.08 ^a
10	18.5	486.11±66.91 ^a	402.15±58.07 ^a	82.28±10.62 ^a
11	12.0	510.06±67.18 ^a	421.69±57.66 ^a	88.38±9.53 ^a
12	9.0	470.69±68.40 ^a	400.68±60.24 ^a	70.02±8.31 ^a

Data of Table 4 were analyzed by using post hoc multiple comparisons ANOVA. Data with different lowercases letters in a row is regarded as significant or very significant differences ($P<0.05$).

the excreta was highest in autumn and lowest in summer. During the same season, the excreta of 4-year adults were smaller than 1-year nonages ($t=6.517$, $df=71$, $P=0.000<0.05$, Table 1, Table 2). There were no significant differences among the excrement calorific values of the 4-year adults ($t=1.024$, $df=71$, $P=0.453>0.05$, $n=72$), and nonages were also no significant differences ($t=1.483$, $df=71$, $P=0.429>0.05$, $n=72$).

Body weight

The body weight of Elliot's pheasant nestling was analyzed by using post hoc multiple comparisons ANOVA. The results (Table 5, Figure 3) showed that the

Table 5 Body weight and average daily increase of body mass of nestlings

Day	Body weight/g	Day	Average daily increase/g
1	21.64±0.54 ^a	1-10	0.66±0.02 ^a
10	28.25±0.58 ^b	11-20	1.07±0.01 ^b
20	38.94±0.56 ^c	21-30	1.59±0.03 ^c
30	54.86±0.24 ^d	31-40	1.74±0.04 ^d
40	72.30±0.25 ^e	41-50	5.99±0.06 ^e
50	132.2±0.42 ^f	51-60	4.24±0.01 ^f
60	174.59±0.42 ^g	61-70	7.88±0.03 ^g
70	253.56±0.58 ^h	71-80	2.09±0.02 ^h
80	274.41±0.48 ⁱ	81-90	6.32±0.22 ⁱ
90	337.56±2.68 ^j	91-100	7.12±0.38 ^j
100	408.76±6.48 ^k	1-100	3.50±0.33
<i>P</i>	0.000	<i>P</i>	0.000

Data of Table 5 were analyzed using post hoc multiple comparisons ANOVA. Data with different lowercases letters in a row is regarded as significant or very significant differences ($P<0.05$).

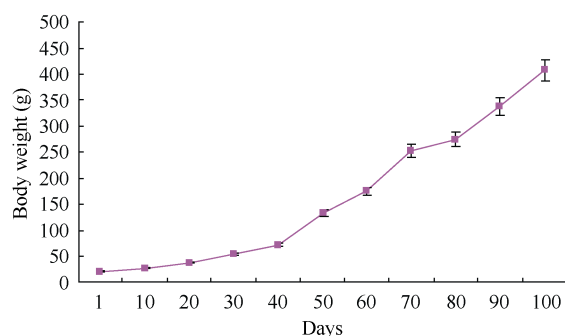


Figure 3 Daily change of nestling body weight

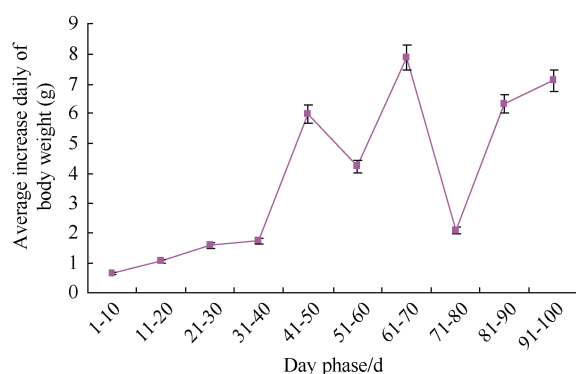


Figure 4 Average daily increase of nestling body mass

average body mass was 22.34 ± 1.69 g at the hatching and the average daily increase of body mass was 7.88 ± 0.03 g, which was the maximum when the nestling was 60–70 days old. The 100 day old nestling was 408.76 ± 6.48 g, close to its adult body mass. After 100 days, the average daily increase of body weight decreased and the body weight gained slowly (Figure 4). The *Syrmaticus ellioti* nestling was close to 300 days when energy intake experiments were measured; each seasonal body weight had no change before and after the formal experiments: 910.80 ± 39.40 g, 925.00 ± 53.40 g, 938.00 ± 33.60 g and 941.20 ± 20.20 g, for spring, summer, fall and winter respectively. Each season the body weight of 4-year adults also had no change before and after the formal experiments: $1\,029.30 \pm 46.60$ g, $1\,017.50 \pm 50.26$ g, $1\,012.40 \pm 42.50$ g and $1\,021.90 \pm 30.00$ g, for spring, summer, fall and winter, respectively.

DISCUSSIONS

Food and food digestibility

The analysis of energy balance of Elliot's pheasant was established on conditions of natural light and cage situations in four different seasons. The foods afforded to the specimens were corns, compound forages and cabbages whose nutrition was very rich, and there was no significant difference for the individual body weight of birds before and after the experiment. Comparing the study results (Table 1, Table 2) with previously reported

results of wild birds' energy metabolism, the food digestibility of Elliot's pheasant is lower than bird eating seeds. This may be one of the reasons that the foods afforded were given artificially and the study of diet selection of Elliot's pheasant have not been previously done. A further study is needed to determine that the impact of variation wild diet of Elliot's pheasant on its food digestibility. The energy metabolism levels of birds can also be affected by their activity (Freckleton et al, 2002). As the trial pheasants exercised in a limited spatial cage, their energy metabolism level were slightly lower than wild bird eating seeds in the natural conditions, but higher than that of the Common Pheasant *Phasianus cochicus* (79.8%), as reported by Wang et al (2003).

Impact of environment temperature on energy intake

Energy metabolism maintains existence when the birds' weight remains constant, thus the metabolic energy is equal to survival energy (Kendeigh, 1975). With increased temperature, the gross energy intake and metabolic energy of Elliot's pheasant declined (Table 1, Table 2) consistent with earlier findings (Olson & Kendeigh, 1980; Zhang et al, 1998). Climate is one of the most important factors that determine individual energy consumption level of species and interspecies (Canterbury, 2002). Here, we showed that the gross energy intake, metabolic energy and excrement energy varied monthly and decreased as temperature increased. At 29.6 °C August had the highest temperature and the metabolic energy was the lowest, while the 5.0 °C of February was the lowest temperature and the metabolic energy was highest for the entire year. With the decreasing temperature, food digestibility decreased, and this is the reason for the increase of gross food consumption at hypothermia. To maintain the birds' energy balance as the temperature increases, the excrement energy decreased, and food digestibility is increases. The energy metabolism level of the birds directly reflects their tolerance at the low ambient temperature; the populations' highly metabolic capacity is more adaptable to the cold environment (Likenes & Swanson, 1996; Swanson, 1995). Our study showed that the food digestibility was the highest in winter, perhaps as an adaptive response to the colder temperatures.

Analysis of excreta, excrement energy and excrement calorific value

In the same age, there were significant seasonal differences for the excreta (4-year adults: $F=72.279$, $P=0.000 < 0.05$, $n=72$; 1-years nonages: $F=23.562$, $P=0.000 < 0.05$, $n=72$) and excrement energy of Elliot's pheasants (4-year adults: $F=16.673$, $P=0.000 < 0.05$, $n=72$; 1-year nonages: $F=97.491$, $P=0.000 < 0.05$, $n=72$). Excreta were the largest in autumn, but in summer the

smallest. Altering the form of digestive tract or widen the volume of it may be an adaptive response to alter the form of digestive tract or widen the volume of it (Gross *et al.*, 1985), and the adaptive capacity of the bowel determines the maximum rate of food digestibility (Kooyman *et al.*, 1992) may be adaptive responses. In the same season, the excreta of 4-year adults were smaller than that of 1-year old nonages (Table 1, Table 2). This may be relative to the diversity of the structure of digestive tract. Elliot's pheasant can tolerate the cold in winter and adapt to the environment by decreasing excreta, thereby increasing energy digestibility. There were no significant differences among the excrement calorific values of Elliot's pheasant, but the trend of decreasing as temperature increased is in accordance with Wang's (1996) earlier findings.

Impact of body weight and age on energy digestibility

The energy metabolism level of bird depends on their body weight (McNb, 2000). There were 2 groups of Elliot's pheasants (4-year adults and 1-year nonages) in this experiment. The average body weight of 100 days old nestling hatched in May 2010 was 408.76 ± 6.48 g, close to adult body weight. After 100 days, the average daily increase of body weight decreased and the body weight grew slowly (Figure 4). When nestling were 300 days old, each seasonal body mass had no change before and after the formal experiments; 910.80 ± 39.40 g, 925.00 ± 53.40 g, 938.00 ± 33.60 g and 941.20 ± 20.20 g, in spring, summer, fall and winter, respectively. The average body weights of 4-year adults in spring, summer, fall and winter were 1029.30 ± 46.60 g, 1021.90 ± 30.00 g, 1017.50 ± 50.26 g and 1012.40 ± 42.50 g, respectively. In the same season, the energy digestibility of 4-year adults was stronger than 1-years nonages, as digestibility may be affected by body weight.

The Elliot's pheasants hatched in May 2010, less than one year old, are in the growth and development phase but tend to slow as they age, and their average body weights were less than that of the 4-year old adults. The large individual animals have relatively small body surface areas, and heat loss of per unit body weight is relatively less in winter; however, in the conditions of

high temperature during summer, the heat of environment flow into animal's body is relatively small (Luo *et al.*, 2008), so the needs of gross metabolic energy of the per unit time per unit body weight of Elliot's pheasant is less, and energy digestibility is stronger. The results indicate that the heavier birds had stronger food digestibility, in accordance with research done on the Mountain Finch *Leucosticte brandti* (Qian *et al.*, 1983). We also discovered that female Elliot's pheasant in the 4-year adults group had a higher average metabolic energy than the males per day, but their body weight was lower than the males. The reasons for this discrepancy were that the female Elliot's pheasant of 4-year adults had to accumulate more adipose than males for reproducing in next year, and the female bird in a semi-domesticated state can maintain spawning for a relatively longer time by redistributing nutrients in their body (Zhang & Yang, 2005), so the use of individual additional energy is concerned with the increase of total adipose (Wise & Weight, 1994).

From the above analysis, we can infer that there is significant difference between the gross energy intake, excrement energy, metabolic energy and temperature of different age groups of Elliot's pheasant. At the same time, body weight and age most certainly impact food digestibility during the same season, food digestibility was higher in of 4-year adults than in 1-year nonages. Zhou (1990) reported that the food digestibility of wild birds taken seeds or nuts were $(89.5\% \pm 4.7)\%$. Our results showed that the average food digestibility (%) of 4-year adults and juveniles were $(85.44 \pm 0.54)\%$ and $(84.14 \pm 0.55)\%$, respectively. Elliot's pheasant mainly eat plant leaves, stems, buds, flowers, fruits, seeds and other plant food crops; it also eats insects and other animal food (John *et al.*, 2000). The metabolic energy of the two groups of trial pheasant were the lowest in August (29.6°C), suggesting that Elliot's pheasant can better adapt to subtropical mountain environments through good food digestibility.

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Blockage of glucocorticoid receptors during memory acquisition, retrieval and reconsolidation prevents the expression of morphine-induced conditioned place preferences in mice

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Abstract: Association between the reward caused by consuming drugs and the context in which they are consumed is essential in the formation of morphine-induced conditioned place preference (CPP). Glucocorticoid receptor (GRs) activation in different regions of the brain affects reward-based reinforcement and memory processing. A wide array of studies have demonstrated that blockage of GRs in some brain areas can have an effect on reward-related memory; however, to date there have been no systematic studies about the involvement of glucocorticoids (GCs) in morphine-related reward memory. Here, we used the GR antagonist RU38486 to investigate how GRs blockage affects the sensitization and CPP behavior during different phases of reward memory included acquisition, retrieval and reconsolidation. Interestingly, our results showed RU38486 has the ability to impair the acquisition, retrieval and reconsolidation of reward-based memory in CPP and sensitization behavior. But RU38486 by itself cannot induce CPP or conditioned place aversion (CPA) behavior. Our data provide a much more complete picture of the potential effects that glucocorticoids have on the reward memory of different phases and inhibit the sensitization behavior.

Keywords: Addiction; Conditioned place preference; RU38486; Glucocorticoid receptor; Retrieval; Reconsolidation; Reward memory

Opiate addiction is a chronic brain disorder characterized by compulsive drug seeking and accompanied by a high risk of relapse, even after long periods of abstinence, which is often precipitated by drug-associated cues (De Vries & Shippenberg, 2002). These environmental cues are paired with the reward from opiate exposure and together shape addictive memory (Valjent et al, 2006). At present, numerous studies indicate that opiate exposure can pathologically usurp neural mechanisms of learning and memory, even under normal circumstances (Hyman et al, 2006).

Conditioned place preference (CPP) has commonly been applied to study the effects of various drugs on reward-related memory (Bardo & Bevins, 2000). In this procedure, drugs are repeatedly administered paired with

a particular environment. When subjects have obtained addictive memory, the drug-paired environments elicit drug-seeking behavior in a drug free drug state (Bardo & Bevins, 2000). If the animal stays longer in drug-paired environments than in non drug-paired environments, that drug-paired environments could be said to have acquired a conditioned incentive property. In CPP training, the reward-related memories consist of acquisition, retrieval and reconsolidation phases. During acquisition, animals receive the drug or saline in an appropriate, distinctive

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chamber and then form memories about the drug and cues. In the retrieval phase, cues previously associated with the receipt of drugs strongly induce animals to reinstate drug-seeking and drug-taking behavior (Lai et al, 2008). Afterward, the reconsolidation phase that follows memory retrieval begins once a previously consolidated memory is activated by conditioned cues, causing memory to enter a labile state that is vulnerable to disruption (Nade, 2003).

Glucocorticoid receptors (GRs) are highly expressed in the olfactory tubercle, nucleus accumbens, striatum, amygdala, hippocampus, hypothalamus and, to a larger extent, areas of the cerebral cortex (Pesini et al, 1998). Glucocorticoid (GC) hormone can mediate a variety of nervous functions including pain, mood, reward, cognition and affect (Wolkowitz et al, 2009). A previous study found that the administration of corticosterone was able to precipitate the reinstatement of cocaine self-administration (Deroche et al, 1997). Adrenalectomy significantly blocked the potentiation of CPP to morphine induced by previous inescapable shock (Der-Avakian et al, 2005). Intermittent exposure to foot shocks delayed the extinction of CPP (Wang et al, 2006), and reinstated morphine CPP after extinction (Wang et al, 2006). Some studies, however, reported that blockage of GR using RU38486 in the hippocampus and nucleus accumbens (NAc) impaired the formation of morphine CPP in Sprague-Dawley rats (Dong et al, 2006). But, GRs blockage's effects on memory and motivation have not reported.

To systemically examine precisely how GR blockage affects reward memory and motivation behavior, we used the GR antagonist (RU38486) and the CPP/ sensitization model in mice and examined if blockage of GRs could pharmacologically affect the acquisition (Experiment 1), retrieval (Experiment 2) and reconsolidation (Experiment 3) of reward-related memory in CPP. Furthermore, we also tested whether RU38486 can affect sensitization behaviors (Experiment 5).

METHODS AND MATERIALS

Animals

Male ICR mice (Animal Center, Kunming Medical College, weighing 20-25 g, 8 weeks of age) were housed in plastic cages with free access to food and water (8 mice/cage) at room temperature [(25±1) °C under a 12 h light/dark cycle (white lights on: 08:00-20:00)]. All animals were treated in accordance with the guidelines of the National Institutes of Health on principles of animal care. Animal facilities and experimental protocols adhered to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Each of the following experiments used an independent set of animals.

Drugs

RU38486 (Zhejiang Xianju Pharmaceuticals Company) or its vehicle (0.9% saline) were given as a single dose via intraperitoneal (i.p.) injection. Morphine hydrochloride was purchased from the First Manufactory of ShenYang, China.

Apparatus and behavioral experiments

CPP Apparatus: Tests were conducted in identical rectangular enclosures of equally sized wooden boxes with compartments (30.7 cm length×31.5 cm width×34.5 cm height) separated by a gray start compartment (13.8 cm length×10.5 cm width×34.5 cm height) with two removable, black guillotine doors. The two compartments had different visual features (horizontal or vertical black and white stripes, placed at equal intervals) and distinct floor textures (smooth in the horizontally striped compartment and rough in the vertical stripes compartment) (Tilley et al, 2007). A video camera was suspended from the ceiling to record the location of each mouse. The time spent in each compartment was manually analyzed offline.

CPP Behavioral procedure:

The experiment included a pretest, conditioning, and test phase. All subjects were weighed and briefly handled daily prior to the CPP procedures and all CPP studies were conducted during the same time of day.

From days 1-3, mice were preconditioned for the morphine place preference procedure as follows. Animals were placed in the central compartment with both doors open to freely explore the apparatus for 15 min per day. All compartments were cleaned and wiped dry between animal runs. After three days of preconditioning, mice were determined for initial preference on day 3. Animals showing a strong unconditioned aversion (less than 30% of session time) or preference (more than 70% of session time) for any compartment were discarded (11 mice were discarded in total) (Carey et al, 2005). From days 4-7, mice were conditioned by the administration of morphine to the non-preferred side and saline on preferred side. Mice were treated for eight consecutive sessions with alternating injections of morphine (40 mg/kg, i.p.) or saline (1 mL/kg, i.p.) during the training period (Tan et al, 2007). The injection time order (morning or afternoon) was counterbalanced with 6 hours intervals between morphine and saline (Song et al, 2007; Do Couto et al 2003). Thus, every animal received both a morphine and saline injection each day.

The test day for morphine-induced CPP was done 24 h after the last conditioning day. Each animal was placed into the central compartment and had free access to all compartments in the 15 min test period. During the test, the animals' time spent in each compartments was

recorded, including expression, extinction and reinstatement. Expression: The CPP expression test took place 24 h after the last conditioning day. Mice were placed in the center compartment in a drug-free state and allowed to explore the entire chamber for 15 min while we recorded the time spent in each compartment. Extinction: After CPP expression, mice were not given extinction test once a day until CPP extinction. No injections were given during the extinction period (Bahi et al, 2008; Bilbao et al, 2008; Mueller & Stewart, 2000). If the time spent in the drug paired side was not significantly different from the time spent in the no drug paired side, CPP were deemed extinguished. Reinstatement: All mice were tested for the reinstatement of CPP immediately after receiving a priming injection of morphine (2.0 mg/kg, i.p.) or saline (0.1 mL/kg, i.p.) The day following the last extinction trial. During this reinstatement test, animals were allowed to freely access to the entire apparatus for 15 min, and the time spent in each chamber was measured.

Locomotion activity: Locomotive activity in wooden cages (0.35 m×0.35 m×0.3 m) was recorded by automatic tracking system with computer-monitored infrared detector.

Experimental Design

Experiment 1: Effects of RU38486 on acquisition of morphine CPP

Experiment 1 was performed to examine the effects of RU38486 on the acquisition of morphine reward-related memory. Morphine CPP conditioning was identical to that in the previous experiments (Tan et al, 2007). After preconditioning, either different doses of RU38486 (10, 20, and 30 mg/kg, i.p.) or vehicle were administered 15 min prior to morphine or vehicle. On day 8, all groups received a drug-free preference test at 15 min. The experimental design for experiment 1 is illustrated in Figure 1A.

Experiment 2: Effect of RU38486 on retrieval of morphine CPP

Experiment 2 was designed to determine whether the ability of RU38486 disrupted the retrieval of morphine CPP. After CPP training as described above, animals were tested for CPP expression on day 8 (post-conditioning). Then, retrieval of reward memory was tested on days 9, 12, 15, and 18. Mice were administered RU38486 (10, 20, and 30 mg/kg, i.p.) or vehicle 15 min prior to every retrieval tests. The experimental design for experiment 2 is illustrated in Figure 2A.

Experiment 3: Effect of RU38486 on reconsolidation of morphine CPP

Experiment 3 was conducted to determine the

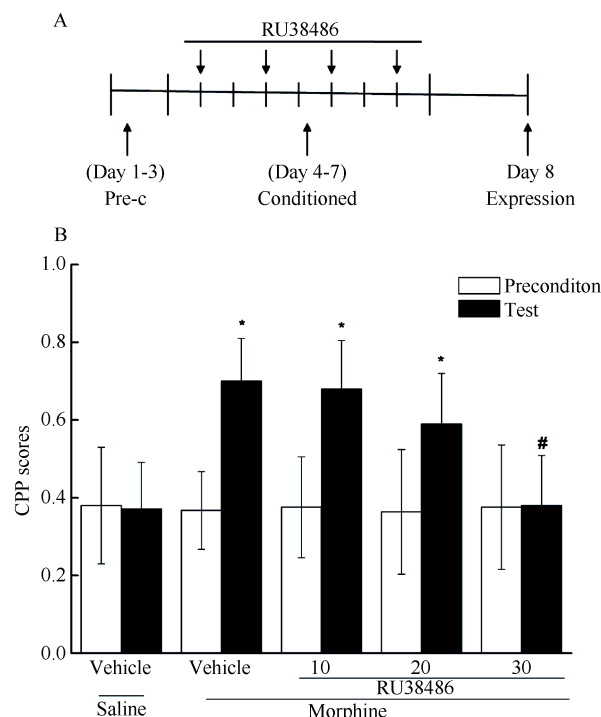


Figure 1 Effects of RU38486 on the acquisition of morphine reward-related memory

A: Behavioral procedure; B: RU38486 disrupted the acquisition of morphine reward memory.

A significant difference was found for post-conditioning CPP scores contrasted with precondition scores in morphine group (morphine-vehicle), 10 RU38486 group (morphine-10 mg/kg RU38486, $P < 0.05$), and 20 RU38486 group (morphine-20mg/kg RU38486, $P < 0.05$). However, no significant difference was found for post-conditioning CPP scores contrasted with precondition scores in 30 RU38486(morphine-30 mg/kg RU38486) group ($P > 0.05$). *: a significant difference contrasted with precondition score ($P < 0.05$); #: a significant difference contrasted with morphine (morphine-vehicle) group ($P < 0.05$); Data ($n=8$) are expressed as mean±SEM.

effects of RU38486 on the reconsolidation of morphine reward memory. Mice were trained for morphine-induced CPP and reconsolidation of reward memory was done in drug-paired compartment for 45 minutes (Milekic et al, 2006). Immediately after the reconsolidation session, different doses of UR38486 or vehicle were injected to investigate the effects on memory reconsolidation. Mice received one of the following treatments: vehicle, RU38486 (10 mg/kg, i.p.), RU38486 (20 mg/kg, i.p.) and RU38486 (30 mg/kg, i.p.). After 24 h, reconsolidation of reward memory in 15 min was test for each group. The experimental design for experiment 3 is illustrated in Figure 3A.

Experiment 4: Rewarding or aversive effects of the RU38486 per se in mice

To rule out the possibility that RU38486 has a rewarding or aversive effect, in experiment 4 we tested whether CPP or CPA behavior could be induced by

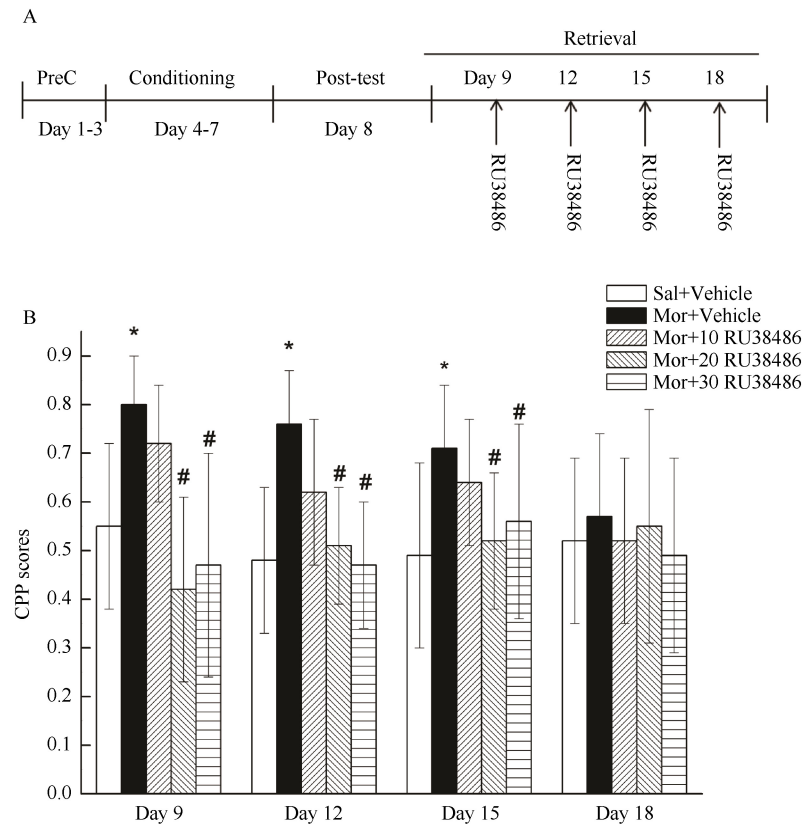


Figure 2 Effect of RU38486 on the retrieval of morphine reward-related memory

A: Behavioral procedure; B: RU38486 disrupted the retrieval of morphine reward memory. *: the vehicle-treated animals that received morphine were significantly different than the control group ($P < 0.05$); #: the groups treated with RU38486 were significantly different than the morphine group (vehicle-treated animals that received morphine, $P < 0.05$); Data ($n=8$) are expressed as mean \pm SEM.

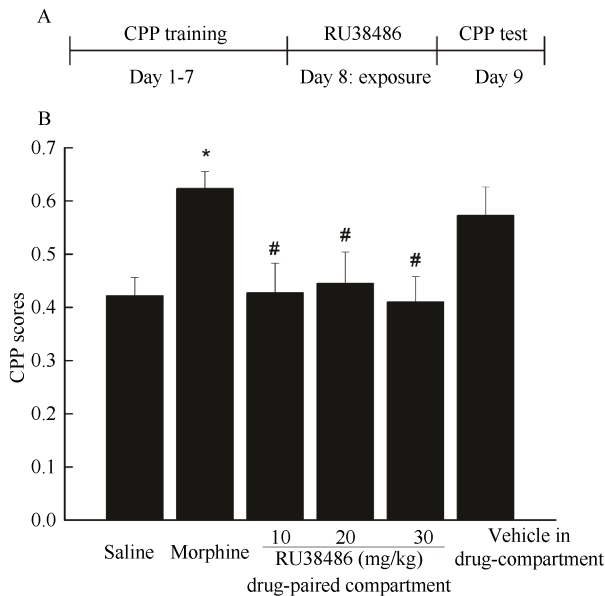


Figure 3 Effects of RU38486 on the reconsolidation of morphine reward-related memory

A: Behavioral procedure; B: RU38486 disrupted the reconsolidation of morphine reward memory. A significant difference was found 24 h after reconsolidation between the vehicle group and RU38486 treatment (10, 20 and 30 mg/kg); *: $P < 0.05$ compared with morphine group (vehicle-treated animals that received morphine); Data ($n=8$) is expressed as mean \pm SEM.

RU38486 alone. Mice were randomly divided into four groups, of 16–20 mice in each group: the vehicle group, 10RU38486 (10 mg/kg) group, 20RU38486 (20 mg/kg) group and 30RU38486 (30 mg/kg) group. After the preconditioning phase, every group was further divided into two equally sized groups. For half of the mice, the smooth compartment was paired with different doses of RU38486, and the rough compartment was paired with saline (0.1 mL/animal). For the other half of the mice, the rough compartment was paired with the above doses of RU38486 and the smooth compartment with saline. The training lasted for three days. During the testing (day 8), the time spent in the two compartments was recorded.

Experiment 5: Effects of RU38486 on the morphine-induced sensitization

To determine the effects of RU38486 on the development of morphine-induced sensitization, the mice were pretreated in open-field cages for five consecutive days. Before pretreatment sessions, the mice were first allowed to habituate to the open field for 30 min in three consecutive days and were then divided into control group (saline-vehicle), morphine group (morphine-vehicle), 10 RU38486 group (morphine-10 mg/kg RU38486), 20 RU38486 group (morphine-20 mg/kg

RU38486) and 30 RU38486 group (morphine-30 mg/kg RU38486). At 15 min after the administration of RU38486 or vehicle, the mice were injected with either morphine (40 mg/kg, i.p.) or saline to sensitization conditioning. The pretreatment dose of morphine used was based on pilot studies. At 30 days after the last pretreatment session, the mice were challenged with morphine (5 mg/kg) (Cordonnier *et al.*, 2007). The locomotor activity of animal was tested after 30 min.

Statistical analysis

During the CPP testing phase, the amount of time spent by the mice in the chambers was recorded by a computer, and preference scores determined by the time spent in the drug-paired compartment divided by the total time in both compartments. To determine whether trained mice exhibited a significant conditioned place preference or aversion, preference scores were contrasted using repeated measures ANOVA with the day-factor as the within-groups variable and group-factor as the between-groups variable. This analysis was followed by between-group comparisons, which were completed with a post hoc analysis (LSD) test in SPSS 13.0 (Gerdjikov & Beninger, 2006). All behavioral data were collected from videorecordings was obtained by an investigator blind to the experimental conditions that performed off-line manual scoring.

RESULTS

Effect of RU38486 on the acquisition of morphine reward memory

In order to determine the involvement of GRs activity in acquisition of morphine CPP, RU38486 or vehicle was administered the 15 min prior to morphine or saline exposures during each conditioned trial. Repeated measures ANOVA revealed that a significant main effect ($F_{(4,31)} = 6.089$, $P = 0.001$) was found after conditioned training contrasted with preference score of precondition (Figure 1B). Post hoc analysis (LSD) then showed that the morphine group as compared with the control group spent more time in the drug-paired compartment ($P = 0.003$). Oppositely, the RU38486-treated animals (10, 20 and 30 mg/kg) that received morphine showed a significant attenuated preference effects ($P = 0.003$, $P = 0.003$, $P = 0.0005$, respectively) contrasted with vehicle-treated animals. These data showed that RU38486 was able to impair either morphine reward or the memory formation necessary for acquisition of morphine CPP in mice.

Effect of RU38486 on retrieval of morphine reward memory

In order to determine the involvement of GRs activity in retrieval of morphine CPP, RU38486 or

vehicle was administered the 15 min prior to retrieval test. As shown in Figure 2B the repeated measure ANOVA (two-way) was conducted on CPP score using doses of RU38486 (10, 20 and 30 mg/kg) as the between-subjects factors and test (day 9, day 12, day 15 and day 18) as the within-subjects factor revealed a significant effect of dose [$F_{(3,34)} = 3.853$, $P < 0.05$]. There was also a significant interaction of group \times day [$F_{(12,34)} = 2.363$, $P < 0.05$]. Pre-test injection of RU38486 treatment markedly blocked the expression of morphine-induced CPP in mice (20 mg/kg RU38486 group vs. morphine group: $P = 0.016$; 30 mg/kg RU38486 group vs. morphine group: $P = 0.026$). The results of pair-wise comparisons for each day showed that there were still significant differences for the CPP score in the morphine as compare with saline on day 9, day 12 and day 15 test. There was no significant difference on the test conducted on day 18. In apparel, post-hoc tests (LSD) showed that the morphine group had significantly increased scores ($P = 0.008$) as compared with the control group. In general, the above results indicated that blockage of GR substantially attenuates the retrieval of morphine-induced CPP.

Effect of RU38486 on the reconsolidation of morphine reward-related memory

In order to determine the involvement of GRs activity in reconsolidation of morphine CPP, RU38486 or vehicle was administered immediately after memory activity. One-way ANOVA showed a significant difference between groups ($F_{(5,48)} = 2.580$, $P = 0.021$) and post-hoc analysis revealed that, after morphine training, morphine group mice acquired CPP (morphine vs. saline: $P = 0.005$) (Figure 3B). RU38486-treated groups placed in the drug-paired compartment showed significant inhibitory effects on the expression of CPP (morphine vs. 10 mg/kg RU38486: $P = 0.006$; morphine vs. 20 mg/kg RU38486: $P = 0.013$; morphine vs. 30 mg/kg RU38486: $P = 0.006$). However, animals treated with vehicle in drug-paired compartment did not show a significant inhibitory effect on the expression of CPP (morphine vs. morphine vs. vehicle: $P = 0.312$). Together, these results demonstrate the inhibitory effect of RU38486 on reconsolidation of morphine reward-related memory within 24 h and emphasize the importance of reward-related cues.

Rewarding or aversive effects of the RU38486 per se in mice

In order to determine the effect of RU38486 on the rewarding or aversive effects in mice. The effects of i.p. administration of RU38486 (10, 20, 30 mg/kg) on the expression of place preference are shown in Figure 4. In this protocol, whether RU38486 can induce CPP or CPA was tested in the CPP procedure. RU38486 was paired

with the smooth or rough compartment, and the training lasted for 3 days like the morphine CPP procedure. Group scores were compared, and the results showed no differences between the rough ($F_{(3,28)}=0.230$, $P=0.875$) and smooth ($F_{(3,28)}=0.251$, $P=0.752$) groups. These data further indicate that RU38486 cannot induce CPP or CPA behavior in mice at these doses, and that RU38486 has no direct effects on either reward or aversion.

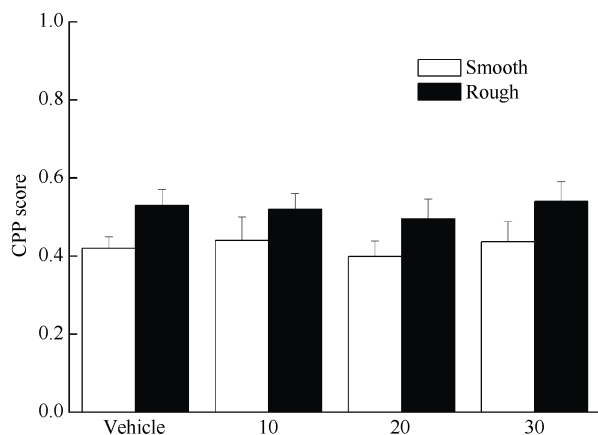


Figure 4 RU38486-induced expression of CPP in mice

The effects of RU38486 alone were tested on the expression of conditioned place preference in mice. Results showed that these three doses of RU38486 do not induce the expression of CPP in mice, independent of whether mice were conditioned in the rough or smooth compartment. Time spent was not different in the smooth or rough compartment per dose. Bars ($n=8$) show mean ± SEM.

Effect of RU38486 on the sensitization in mice

Morphine-induced sensitization during the challenge session is altered by RU38486 pretreatment (Figure 5). One-way ANOVA showed a significant difference between groups ($F_{(4,35)}=6.783$, $P<0.05$) and post-hoc analysis revealed that, after morphine challenged (5 mg/kg), RU38486 inhibited the sensitization behavior (vehicle-morphine vs. 10 RU38486, $P<0.05$; vehicle-morphine vs. 20 RU38486, $P<0.003$; vehicle-morphine vs. 30 RU38486, $P<0.05$). These data showed that RU38486 was able to impair morphine induced sensitization.

DISCUSSION

Our study illustrates that drug associated learning and memory played a key role in drug craving and psychological dependence. The conditioned place preference paradigm has been extensively used in drug addiction research to investigate reward-related learning and memory (Bardo & Bevins, 2000). Here, during conditioning, animals are trained in one environment paired with drugs and another paired with saline. On the test day, animals were freely given access to both environments in a drug-free state and their preferences

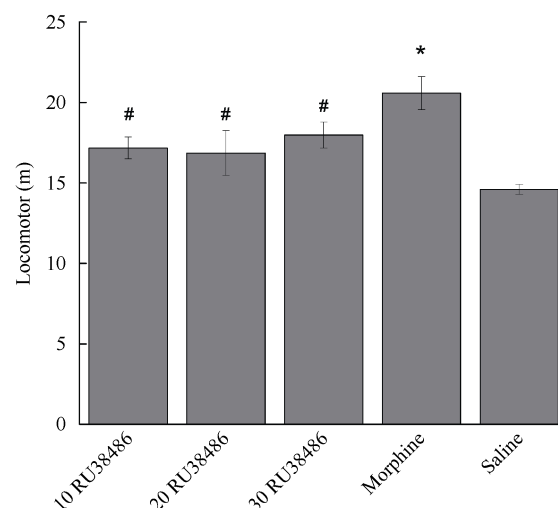


Figure 5 Effect of RU38486 on the sensitization in mice

*: $P<0.05$ compared with control group; #: $P<0.05$ compared with morphine. Bars ($n=8$) show mean ± SEM.

for drug- versus saline-paired environments are assessed, the expression test is thought to be a measure of memory acquisition, retrieval and reconsolidation (Han et al, 2010; Zhai et al, 2008). The current study was designed to examine the role of GRs receptor antagonist, RU38486, in morphine-related reward memory, using the CPP paradigm. Current results show that a) treatment with RU38486 attenuated the expression of reward-related memory in the CPP paradigm included the acquisition, retrieval and reconsolidation; b) RU38486 itself did not induce a conditioned placed preference or aversion; c) RU38486 disturbed the sensitization behaviors of mice. Our results demonstrate that morphine-induced reward memory included the acquisition, retrieval and reconsolidation in CPP model and sensitization behaviors that involve GRs can be destroyed by RU38486.

Based on previous studies, there are three important factors in the CPP procedure. First, the drug has rewarding properties that establish the drug-seeking motivation that drives the animals to establish a place preference (Meng et al, 2009). Second, animals can remember the drug-associated cues, which reactivate the reward-related memories. Both environmental contexts and specific environmental cues associated with a drug consistently seem to be able to reinstate drug-seeking behaviors in abstinent subjects (Shalev et al, 2002). Finally, the associative memories formed in CPP procedures are both stable and persistent. Therefore, treatments attenuating the expression of CPP may act by disrupting any of these three factors.

Piazza & Le Moal (1997) administered drugs, such as opiates and psychostimulants, which could elevate the level of glucocorticoids and DA in NAc and enhance the reward and memory procedure in the CPP model. During

the acquisition of reward memory, blockage of GRs could attenuate the DA response to drug which impaired the conditioned learning. Firstly, DA responses to drugs may be mediated in part by glucocorticoids, as surgical or pharmacological adrenalectomy (without basal replacement) attenuated self-administration and locomotor responses to cocaine as well as the DA response in NAc to morphine (Barrot *et al.*, 2000; Goeders & Guerin, 1996). Previous studies showed that corticosterone-induced dopamine release was further increased (ca. 80% above the baseline) if the hormone was administered in parallel with reward-related activities (Piazza *et al.*, 1996). Treatment with RU38486 during acquisition led to the impaired reward. On the other side, learned association between drugs abuse and context is essential for the formation of drug conditioned place preference (CPP) and glucocorticoid hormones also play an important role in this action. Blockage of GRs can disturb the conditioning learning by RU38486, leading to the impairment of reward memory acquisition. Animal and human studies have also indicated that the administration of low doses of glucocorticoid hormones enhanced memory for stressful or emotionally arousing events (Roozendaal *et al.*, 2009). These enhancing effects depend on the integrity of the amygdala noradrenergic system (Roozendaal *et al.*, 2009). In general, acquisition of reward memory in CPP was perhaps destroyed by the two ways.

Neural circuits of associative learning include several brain regions, such as the hippocampus, and basolateral amygdala, medial prefrontal cortex, orbitofrontal cortices (Dong *et al.*, 2006; Maratos *et al.*, 2001), and GRs are widely distributed in these areas (Piazza & Le Moal, 1997). Riedel *et al.* (1999) addressed the role of the hippocampus in retrieval by training animals to form a spatial preference with normal hippocampal function. When the hippocampus was temporarily inactivated before a spatial memory test, retrieval was impaired (Riedel *et al.*, 1999). Similarly, glucocorticoid effects on memory retrieval were recently reported in human subjects. Oral administration of stress doses of cortisone 1 h before retention testing impaired hippocampal-dependent free recall of previously learned words (de Quervain *et al.*, 2000). These findings demonstrated that once memories are consolidated, the efficacy or accuracy of the information retrieved remains vulnerable to glucocorticoids at the time of recall. Here, the administration of RU38486 15 min given prior to the reward-memory retrieval that served as reactivation sessions and resulted in a significant suppression in morphine-induced CPP in the following test. However, within the brain the mechanism of change in the gene transcriptions and expressions involved by RU38486 are not clear.

Reconsolidation is the process by which previously consolidated memories are stabilized after retrieval. Disruption of reward-related memories in a reactivation-dependent manner is thought to be due to the disruption of memory reconsolidation processes (Zhao *et al.*, 2011). A recent study showed immediately following memory reactivation, intrahippocampal or systemic administration of the GRs antagonist RU38486 in rats that were trained and tested in an inhibitory avoidance task showed a deficit in reconsolidation memory (Nikzad *et al.*, 2011). Other studies found that a matrix metalloproteinase inhibitor (Brown *et al.*, 2009), a β -noradrenergic receptor antagonist (Debiec & Ledoux, 2004) or N-methyl-D-aspartate (NMDA) receptor antagonists (Brown *et al.*, 2008) can also interfere with the reconsolidation of drug-associated CPP memories. These studies showed that disrupting the reconsolidation process of drug-associated memory formation may be a potential target useful in treating drug addiction (Lee *et al.*, 2005; Nader *et al.*, 2000). In the reconsolidation test, systematic administration of RU38486 could impair the reconsolidation of morphine reward memory. On the contrary, Wang *et al.* (2008) reported that the disruptive effect of stress on reconsolidation of morphine related memory was prevented by inhibition of corticosterone synthesis with metyrapone or injections of the glucocorticoid antagonist RU38486 in BLA. The difference between their results and ours may be due to different treatment methodology, different involved brain regions or a different level of glucocorticoid hormones in reconsolidation. Thus, our findings imply that the RU38486 may impair the reconsolidation of reward memory through inactivation of GRs in the whole brain, which may include in the hippocampus as well.

Our study also shows that expression of the GR is a necessary condition for the development of morphine-induced sensitization at the behavioral levels. This finding is important for two reasons. First, sensitization to the effects of a drug is considered a major process in the development of drug abuse, because it should mediate the uncontrollable motivation for the drug that characterizes addiction (Robinson & Berridge, 1993). Second, the results show that the GCs are included in neuroadaptive process. Notably, our previous data indicated that the GRs seemed to be involved in mediating not the basal behavioral effects of morphine, but, selectively, the excessive response to the drug. Similarly, previous report showed that RU38486 (mifepristone) did not block the acute response to cocaine but selectively suppressed cocaine-induced sensitization (De Vries *et al.*, 1996). Our experiments, by using a pharmacological invalidation approach, demonstrate that the GRs are strongly implicated in the development of sensitization.

In conclusion, using the CPP procedure, we found that morphine can induce CPP in mice, whereas RU38486 alone cannot induce CPP or CPA. Treatment with RU38486 prior to morphine conditioning can

attenuate the acquisition, retrieval and reconsolidation of reward memory. Simultaneously, RU38486 also blocked the sensitization behaviors; these results suggest that GRs may play an important role in addiction procedure.

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